2.1 LITERATURE REVIEW

Gramicidins were isolated from *Bacillus brevis* in 1939 and thus antimicrobial peptides from prokaryotes were discovered. Gramicidin showed antimicrobial activity both in vivo and in vitro against many Gram positive bacteria [Dubos 1939; Dubos 1939]. The therapeutic potential of Gramicidin was later seen, when it was found to treat infected wounds on skin of guinea-pig, thus was found suitable for clinical use [Gause and Brazhnikova 1944]. First commercially produced antimicrobial peptide as antibiotic was Gramicidin [Van and Heather 2006]. In 1896 a substance present in wheat flour was found to be lethal to bread yeast [Jago and Jago 1926]. This substance with antimicrobial activity isolated from wheat flour was further isolated in 1942 from *Triticum aestivum* (wheat) endosperm and was found to be a peptide. The growth of a wide range of phytopathogens , like *Xanthomonas campestris* and *Pseudomonas solanacearum* was shown to be inhibited by this peptide [Caleya et.al. 1972]. This peptide belongs to the family of thionins, which are antimicrobial peptides present throughout the plant kingdom [Stec 2006]. In mid 1970s this peptide was named as purothionin [Ohtani 1977; Mak and Jones 1976]. A need for host defence molecules arose with the advent of multi drug resistant microorganisms in early 1960s. Multi drug resistant microbial pathogens gave a realization of the end of the "Golden Age of Antibiotics", thus the works on antimicrobial peptides were found to be very significant [Davies 2006; Katz 2006; Phoenix 2013].

In the year 1984, Garcia-Bustos et al, purified a small peptide Microcin 7 from *Escherichia coli. Escherichia coli* cells possessing the pMccC7 plasmid, in their stationary phase, produce and excrete this peptide in the culture medium [Garcia-Bustos et. al. 1984]. In the year 1985, Garcia-Bustos et al, showed that the enterobateria having evolutionary closeness to *E.coli* were inhibited by this peptide. The mechanism of inhibition by this peptide was by blocking

the protein synthesis mechanism of the target microorganism. Trypsin was used to degrade this peptide and further purification and sequencing of the fragments was carried out, it was found that the ends of the peptide are blocked and it is a linear heptapeptide [Garcia-Bustos et. al. 1985].

In the year 1997, Tailor et al, isolated four antimicrobial peptides from the seeds of *Impatiens balsamina*. These peptides showed a broad spectrum inhibition over a wide range of bacteria and fungi. The peptides were 20 amino acids long, highly basic in nature and were closely related to each other. The peptides named Ib-AMP1, Ib-AMP2, Ib-AMP3 and Ib-AMP4 were the smallest antimicrobial peptides derived from plants till then. These peptides have two intramolecular disulphide bonds formed by four cysteine residues present in their sequence. The cDNA was isolated and characterized; the analysis showed that a single transcript encodes all these four antimicrobial peptides [Tailor et. al. 1997].

In the year 2000, Otvos et al, studied three insect derived short proline-rich antimicrobial peptides i.e. Drosocin [Bulet et. a. 1993], Pyrrhocoricin [Cociancich et. al. 1994] and Apidaecin [Casteels et. al. 1989]. Native and one of the analogues of pyrrhocoricin was seen to protect mice from bacterial infections and hence was proposed as an alternative to the existing antibacterial drugs. It was further proposed that if the bacterial targets of these peptides were known then these modes of actions can be used to design novel set of peptidic or peptidomimetic antibacterial compounds. Castle et al in 1999 showed the killing mechanism of *Escherichia coli* by Apidaecin was shown through the interaction of Apidaecin with bacterial membrane to enter the cell and then following the killing mechanism through interaction with several intracellular targets [Castle et. al. 1999]. In their study Otvos et al labelled analogues of apidaecin, pyrrhocoricin and drosocin with biotin and flouroscein to detect the mechanism of action path followed by peptides through interaction with various biopolymers which are involved in the killing process. Through this process two interacting

proteins from *E.coli* were isolated, DnaK and GroEL. The control experiments showed that DnaK and antibacterial peptides showed specific binding whereas GroEL and antibacterial peptides showed non-specific binding. The studies also showed that the peptides enter into the cell through Lipopolysaccharide mediated cell entry, as two lipopolysaccharide solutions interacts with all these three antibacterial peptides [Otvos et. al. 2000].

In the year 2004, Hsu et al, predicted that the binding of nisin to lipid II enhances its pore forming activity. A precursor of cell wall synthesis is Lipid II and nisin belongs to lantibiotic group and has a pore forming activity. This interaction increases the pore forming activity of nisin by three folds. In this nisin and lipid II interaction the N-terminal amides of nisin interact with the pyrophosphate moiety of lipid II, intermolecular bonds are formed in this interaction. This provides a reason for the lanthionine ring being conserved among various lantibiotics that bind to lipid II [Hsu et. al. 2004].

In year 2005, Xia and Ng, isolated a peptide from multiple cloved garlic bulbs. This showed antifungal and antibacterial activities. It did not show antifungal activity against *Fusarium oxysporum* but it was active against *Mycosphaerella arachidicola.* It showed antibacterial activity against *Fusarium oxysporum.* This was named Alliumin and purified using ion exchange chromatography and affinity chromatography. Trypsin or Chymotrypsin treatment for 30 mins at room temperature and also 1 hour boiling do not destroy the antifungal activity. Proliferation of Leukemia L1210 cells was inhibited by Alliumin. Inhibition of proliferation of hepatoma HepG2 cells , protease activity and ribonuclease activity were not present in Alliumin [Xia and Ng 2005].

In the year 2005, Dennison et al, investigated the antimicrobial activity of an anionic peptide AP1. Staphylococcus aureus was killed by AP1 with an MIC of 3mM. The interaction of AP1 with the vesicles of lipid extract after FTIR spectroscopy revealed that the peptide is present

in α-helical form and increase in the fluidity of lipid. Thus it was shown that Ap1 interacts with Gram positive bacteria through lipid membrane and the killing mechanism follows and the peptide behaves in α-helical peptide against Gram positive bacteria [Dennison et. al. 2005].

In the year 2010, Rajaram et al, isolated bacteriocin from marine environment strain of *Lactobacillus lactis* and showed it to inhibit some microorganisms which are food borne. Some enzymes like Proteinase K and pepsin inhibited the production of bacteriocin, whereas, enzymes like Lipase, RNase, DNase and α-amylase promote production of bacteriocin. The purified bacteriocin was shown to have a molecular weight of 94kDa and it was purified using ion exchange chromatography. This strain was established as probiotic and bacteriocin showed characteristics of food preservation [Rajaram et. al. 2010].

In the year 2010, Marsh et al, identified lantibiotic type 1 novel clusters; using the conserved nature of Lanthionine synthetase enzyme. Total 49 clusters were identified. These predicted clusters were totally novel and before this prediction showed no relation with any lantibiotic production. Their work showed that the lantibiotic gene clusters were much more in number than actually expected. Thus it was predicted that much more antimicrobials can be produced with the help of these gene clusters. [Marsh et. al. 2010]

In the year 2011, Rehman and Kahnum, isolated and further characterized antimicrobial peptides from *Pisum sativum*. Two antimicrobials S4 and S5 having molecular weight 19kDa and 22kDa respectively were identified from seed and two antimicrobials P7 and P8 having molecular weight 10kDa and 11kDa respectively were identified from pod. Their antibacterial activity was tested against many bacteria and *Staphylococcus aureus* was found to be most susceptible towards these. The optimal temperature and pH of the activity of these peptides is 4-25 ℃ and 5-7 respectively [Rehman and Khanum 2011].

In the year 2011, Aliahmadi et al extracted total water soluble proteins from 10 plants seeds. The antibacterial assay was performed on *Escherichia coli, Enterococcus faecium and Staphylococcus aureus*. Densitometry and Tricin-SDS-PAGE were used for determination of amount and molecular weight of proteins. Less than 5kDa molecular weight peptides from two plants were found to be antibacterial activities. The two plants were *Onobrychis sativa Lam*. and *M. sativa* L. These were more active against Gram positive bacteria as compared to Gram negative bacteria [Aliahmadi et. al. 2011].

In the year 2011, Paramasivan et al, predicted two pant defensins; VrD1 and VrD2 from Vigna radiate to prevent the colonization of *Helicobacter pylori* in human gastric mucosa. These peptides were predicted to provide anticancer properties as *H. pylori* is a cause of gastric carcinoma. The proteins of *H. pylori* used in this study were flgS, groL, nikR, ureI and ureH. STRING database was used to study the interaction of proteins. The specified proteins were modelled using MODELLER 9V8 and PSI-BLAST. The docking studies of the above specified plant defensins with the selected proteins gave significant results and thus it was predicted that this interaction will prevent that colonization of *H. pylori* in the human gastric mucosa. [Paramasivan et. al. 2011]

In the year 2011, Baranska-Rybak et al, work on the effect of antimicrobial peptides on the superantigen producing strains of *Staphylococcus aureus*. The effect of antimicrobial peptides and the effect of conventional antibiotics on the superantigen producing strains were compared. TSST-1, SED, SEC, SEB and SEA were the enterotoxins produced by the superantigens. The antimicrobial peptides used for this study were uperin 3.6, temporin A, tachyplesin 3, protegrin 1, lipopeptide, citropin 1.1 and aurein 1.2. Comparison of antimicrobial peptides and conventional antibiotics effects showed that the conventional antibiotics had higher values of MBCs and MICs for the strains producing superantigens as compared to those that do not produce; whereas, this was not the case with the antimicrobial

peptides. Thus, it was concluded that antimicrobial peptides showed significant activity against all the selected strains of Staphylococcus aureus, and had a higher potential to be developed as therapeutic agents against them. [Baranska-Rybak et. al. 2011]

In the year 2012, Venugopal and Mohan et al, docked five virulence proteins of *Staphylococcus aureus* with four antimicrobial peptides using Patchdock server. The chosen virulence proteins of *S. aureus* were 1STE (enterotoxin C2), 2GOM (extracellular fibrinogen binding protein Efb- C), 2NTT (Staphylococcal enterotoxin K), 3DOA (Fibrinogen binding protein) and 3D5S (Efb-c/C3d complex). The antimicrobial peptides selected for study were 1KV4 (Moricin), 1L4A (Sapecin), 2MAG (Magainin) and 2GW9 (Defensin Cryptdin 4). The dockings were analysed and most significant docking results were given by Staphylococcal enterotoxin K and Moricin docked complex [Venugopal and Mohan 2012].

In the year 2012, Joo et al, worked on the effect of Nisin for the treatment of HNSCC (Head and neck squamous cell carcinoma). Nisin is shown to induce apoptosis and reduce tumorigenesis in HNSCC cells. The mechanism shows that nisin follows the path of checking the proliferation of HNSCC cells through CHAC1. CHAC1 is a proapoptotic cation transport regulator. This process is accompanied by influx of extracellular calcium. They were the first to report the role of CHAC1 during promotion of cancer cell apoptosis with nisin. These studies show that nisin can be a potential candidate for the treatment of HNSCC. As nisin is also used as food preservative, hence it is safe for human consumption [Joo et. al 2012].

In the year 2014, Dai et al, worked on QSAR analysis of peptides and developed an improved pipeline. They used double selection procedure for before final regression analysis- feature selection and sample selection. 531 descriptors were used which were the physicochemical parameters of amino acids. SVR along with weighted near neighbour training sample was used for QSAR model. This pipeline had advantage over single selection procedure as both

sample and feature could be selected in this model. This model could also be very well applied in regression predictions [Dai et. al. 2014].

In the year 2014, Jagannath et al, stabilization of functional vegetable and fruit juices by nisin. The bacterial cellulose of *Acetobacter xylinum* was used. The juices under study were beet root, tomato, musk melon, carrot, pineapple and a mixed juice of 60% beet root and 20% each of tomato and carrot. These juices were stored at low pH, at 28℃ for 90 days without chemical preservatives. The colours were correlated with the pigment concentration present in stored and fresh juices. The studies revealed that after 90 days storage total carotenoids concentration in the case of pineapple, carrot and musk melon was found to be most for pineapple and least for carrot. Lycopene concentration remained stable. The betanin concentration was found more in the case of beet root juice mixed with tomato and carrot as compared to that of beet root alone [Jagannath et al 2014].

In the year 2015, Oliveira Junior et al, studied the antimicrobial activity of nisin and physiochemical properties of different fruit juices. The juices considered were cupuassu, guava, orange, passion fruit, mango, peach, soursop and cashew. After 30 days study nisin was not shown to cause any modification in the physicochemical characteristics of the juices and nisin itself remained stable. The concentration of Vitamin C was supported by the presence of nisin in the juices. The antimicrobial activity of nisin in the juices was checked against these microorganisms; *Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus and Alicyclobacillus acidoterrestris*. The studies of antimicrobial activities revealed that *Listeria monocytogenes* was least susceptible to the presence of nisin, whereas, *Alicyclobacillus acidoterrestris* was most susceptible. Heating of juices for prevention of microorganisms destroys its organoleptic characteristics. Thus, nisin can be used a better alternative for fruit juice processing. [de Oliveira 2015]

In the year 2015, Wang et al, predicted smaller peptides were more stable in the colon. 17 peptides were used for its stability in a mixture of human faecal bacteria; this mixture was used as a model for large intestine environment. The selected peptides were goserelin, deslorelin, [D-Ser4]-gonadorelin, histrelin, buserelin, nafarelin, leuprolide, ciclosporin, octreotide, Arg-vasopressin, oxytocin, desmopressin, somatostatin, secretin, glucagon, calcitonin and insulin. It was seen that the larger peptides degraded more rapidly as compared to the smaller peptides. It was also shown that in control without the presence of faecal bacteria; all the peptides remained stable. This study reveals the effect of the colonic environment on the effect of peptides [Wang et al 2015]. In the year 2015, Wang et al, studied the effect of the environment in small intestine fluids and gastric fluids on the peptides drugs. The selected peptides were goserelin, deslorelin, [D-Ser4]-gonadorelin, histrelin, buserelin, nafarelin, leuprolide, ciclosporin, octreotide, Arg-vasopressin, oxytocin, desmopressin, somatostatin, secretin, glucagon, calcitonin and insulin. The fluids of both human and pigs were considered for the study. It was seen that the smaller peptides were more stable in gastric fluids. In small intestine cyclic peptides were most stable [Wang et. al. 2015].

In the year 2016, Xu et al, cloned an anionic antimicrobial peptide from *Plutella xylostella* and characterized it. The sequence analysis of the peptide revealed the presence of six cysteine residues and the isoelectric point was 5.57. The peptide was plant defensin and named as PxDef. This was the first reported insect anionic defensin. The mature recombinant peptide has more activity against Gram-positive bacteria. The MIC value of B. thuringiensis was lesser than Bacillus subtilis. Thus it is seen that PxDef provides immunity to *Plutella xylostella* against *B. thuringiensis* [Xu et al 2016].

In the year 2017, Jabeen et al, isolated peptides from *Momordica charantia L*. The isolated peptides were isolated from various parts of the plant. The antibacterial activity was of

extracts of various parts of plant were tested on *Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus* and *Escherichia coli*. The antibacterial activity was observed only in seed extract. The peptides were precipitated and analysed using gel filtration chromatography and molecular mass determined through SDS-PAGE. One peptide having molecular weight ~10kDa was observed. This peptide was active between pH 5-7 and stable between temperatures 4-50 ℃. *S. aureus* was the most sensitive strain among all the four bacteria used in the study. [Jabeen and Khanum 2017]

2.2 THESIS HYPOTHESIS

The literature review gives us the information regarding the threat posed by the emergence of resistant microorganisms, as well as, how these resistant microorganisms will make the existing drugs ineffective. Thus, the diseases that are curable today might become incurable in the near future, as the microorganisms will become resistant against those drugs. Presently, a lot of work is been carried out to develop various antimicrobial peptides as alternate drugs. *In silico* interaction studies of various virulence proteins, transcription activators, etc. with small length antimicrobial peptides could be carried out and potential antimicrobial peptide can be selected. Further studies can be carried out for the development of this potential antimicrobial peptide as alternate drug.

2.3 OBJECTIVES

The increase in the drug resistant bacteria has promoted the Scientists towards the development of alternate drug. These drugs should be such that the microorganisms will have difficulty to develop resistance against them. The research work was carried out to select a potential antimicrobial peptide to be developed as alternate drug. The research work was divided in following phases-

The **first phase** of work involved the docking interaction studies between the toxins of *Staphylococcus aureus* and the antimicrobial peptides of plant origin. The antimicrobial peptide predicted to be the best among these will be considered for further studies.

The **second phase** of work involved the *In silico* interaction studies of the selected antimicrobial peptide with the Transcription activator ToxT of *Vibrio cholerae*.

The **third phase** of the work involved the selection of short length antimicrobial peptide. The *In silico* interaction studies of antimicrobial peptides against the catalytic domain of Diphtheria toxin were carried out.

The **fourth phase** of the work deals with the *In silico* interation studies of the selected antimicrobial peptide with the Transcription activator ToxT of *Vibrio cholerae*.

The **fifth phase** of the study narrates the *In silico* interation studies of the selected antimicrobial peptide against the "Superbug" New Delhi Metallo-β-lactamase-1 protein.

The **last phase** of the work was about synthesis and checking antibacterial activity of the predicted antimicrobial peptide.