INTRODUCTION

Antibiotics have been in use for the past 60 years to fight against various infectious diseases caused by bacteria and other microbes. The disease causing microbes have become resistant to antibiotics due to its increased use and misuse in medicine. About 70 percent of the bacteria that cause infections are resistant to at least one of the drugs most commonly used for its treatment. Certain organisms like methicillin resistant *Staphylococcus aureus*, vancomycin resistant *Enterococci* have developed multidrug resistance. Antimicrobial resistance is a complex issue, resulting largely from the over use or inappropriate use of antibiotics, the spread of drug resistant organisms and shortage of new antibiotic drugs.

The medical and economic impact of antibiotic resistance is huge. In Europe, it is estimated that at least 25,000 people die each year from infections due to antibiotic resistant bacteria, which also result in around 2.5 million extra hospital days. In the United States, every year, methicillin resistant *Staphylococcus aureus* (MRSA) infections alone kill nearly 19,000 people a year which is comparatively higher than AIDS (Klevens, 2007). A recent study has calculated annual costs of antibiotic resistant infections to be in excess of $20 billion(Roberts, 2009).

Resistance is now exploding in developing countries, linked to antibiotic misuse, including lack of patient adherence to prescribed treatment, over-the-counter antibiotics and counterfeit products. Common bacteria such as *Escherichia coli* are now producing enzymes (such as KPC and NDM-1) which make the bacteria to be resistant to almost all antibiotics, including carbapenems, often considered to be the last line of defense in antibiotic treatments.

The important multi drug resistant organisms (MDROs) are methicillin-resistant *Staphylococcus aureus*(MRSA), Extended Spectrum β-Lactamase (ESBL) *Enterobacteria*, Vancomycin Resistant *Enterococci* (VRE) and *Clostridium difficile*. It is widely accepted among clinicians, medical researchers, microbiologists and pharmacologists that antibiotic resistance will, in the very near future, leave healthcare professionals without effective therapeutics for bacterial infections. As an example, it is now estimated that about half of all *Staphylococcus aureus* strains found in many medical institutions are resistant to antibiotics such as methicillin. Recently, a new methicillin resistant *staphylococcus aureus* bacterium has evolved namely
NDM-1 (NewDelhi Metallo-beta-Lactamase). These bacteria produce an enzyme called metallo-beta-lactamase-1 (MDM-1), which is resistant to most of the antibiotics currently available in the market. The emergence among Enterococci resistance to another useful and widely effective antibiotic, vancomycin might accelerate the spread of vancomycin resistant genes, via plasmids, throughout other species, eventually limiting the efficacy of this drug. Consequently, the priority for the next decades should be focused in the development of alternative drugs and the recovery of natural molecules that would allow the consistent and proper control of pathogen-caused diseases. Ideally, these molecules should be as natural as possible, with a wide range of action over several pathogens, easy to produce, and not prone to induce resistance (Zhang and Falla, 2006).

Methicillin resistant Staphylococcus aureus (MRSA) is the most problematic bacteria in public health which comprises approximately 40% of all Staphylococcus aureus. It is resistant to numerous antibiotics of the β lactam family, including methicillin, penicillin and other antibiotics except vancomycin and teicoplanin (Witte, 1999). Vancomycin and teicoplanin are glycopeptide antibiotics used to treat MRSA infections. The resistance to methicillin was due to a penicillin binding protein, PBP 2A, coded by a mobile genetic element termed the methicillin resistant gene (Mec A). In recent years, the gene has continued to evolve so that susceptibility of many MRSA strains to vancomycin has decreased and vancomycin intermediate and vancomycin resistant Staphylococcus aureus have dramatically increased in many countries (Hiramatsu et al., 1997). A decrease in the susceptibility of MRSA to teicoplanin has also been reported in hospitals around the world (Mainardi et al., 1995). The evidence of MRSA resistance to vancomycin and teicoplanin, which are antibiotics of last resort, makes the need for alternative antibiotics and chemotherapeutics.

The incidences of fungal infections have increased significantly in past twenty years due to increase in number of people whose immune system is compromised by AIDS, organ transplantation and cancer therapy. This phenomenon is aggravated by the rapid development of drug resistance against most of currently used antifungal drugs such as fluconozole, itraconozole and amphotericin B. The toxicity of few available antifungal agents and development of resistance during treatment in others are becoming a major problem in the management of current antifungal treatment. The development of new antifungal agent with different mechanism of action and acceptable toxicity is thus urgently needed (Monica Benincasa et al., 2006)
Numerous chemotherapeutic drugs have been developed to treat cancers, including DNA alkylating agents, anti metabolites and hormone agonists/ antagonists. Although these drugs have been successfully used for the treatment of metastatic cancer, severe side effects and dose limitations are prevalent. As a result of their inability to distinguish between cancer cells and proliferating normal cells, current drugs kill both. Cancer cells develop resistance to these drugs that is mediated by the over expression of the multidrug resistant proteins that pump the drug out of the cells and thus render the drugs ineffective (Fez Tomas, 2006). The development of new classes of anticancer drugs that lack the toxicity of conventional chemotherapeutic agents and are unaffected by common mechanism of chemo resistance would be a major advantage in cancer treatment.

Antimicrobial peptides (AMPs) have become as a potential antibiotics due to their killing ability against a wide spectrum of microorganisms. Unlike currently available conventional antibiotics, which typically interact with a specific target protein, most of these cationic AMPs target the cell membrane of invading microorganisms leading to cell lyses and death (Chan et al., 2006; Durr et al., 2006)

Thus, AMPs offer the possibility of a new class of therapeutic agents which are complementary to existing antibiotics and to which bacteria may not be able to develop resistance. AMPs are important components of innate defenses of all species of life (Boman, 1995). AMPs are structurally diverse group of molecules with 12-50 aminoacids which share cationic and amphipathic properties. They have a net charge of +2 to +7. They are found in all species of life from bacteria to mammals (Hancock and Chapple, 1999). According to the nature of synthesis the AMPs fall into 2 classes
1. Nonribosomally synthesized peptides
2. Ribosomally synthesized peptides

**Nonribosomally Synthesized Peptides**

It contains non-protein aminoacid such as D-aminoacids or hydroxyl aminoacids and other aminoacid constituents that undergo extensive modification including N-methylation, acylation and covalent linkage to other functional group. These peptides are found in bacteria, fungi and
streptomyces. Many of the antibiotics like penicillin and polymyxin used in our society are non-ribosomally synthesized peptides (Pristovsek and Kidric, 1999).

**Ribosomally Synthesized Peptides**

The ribosomally synthesized peptides undergo post translational modification and proteolytic processing. These peptides like nisin and subtilin are found in all domains of life (Entian and Devos, 1996).

**Classification of Antimicrobial Peptides**

The AMPs discovered so far have been divided into several groups based on their length, secondary and tertiary structure, amino acid composition and their size and presence or absence of disulfide bridges. Nuclear magnetic resonance (NMR) has emerged as a useful technique for studying details of structures of most of the known antimicrobial peptides (Reddy *et al*., 2004). It is common to classify the peptides broadly into four major groups by their secondary structure (Vant Hof *et al*., 2001). They are

(a) Peptides that form α-helical structures,
(b) Peptides consisting of β-sheets, connected by intra molecular disulfide bridges,
(c) Peptides with extended structures, characterized by over representation of one or more amino acids
(d) Peptides composed of rare and modified amino acids.

**a) Peptides that form α-helical Structures**

One of the larger and better studied classes of antimicrobial peptides is those forming amphipathic helices. These peptides have disordered structure in aqueous solution while fold into a α-helical conformation upon interaction with hydrophobic solvents or lipid surfaces. α-helical peptide are often found to be amphipathic can either absorb on to membrane surface or insert in to membrane surface as helical bundles. The majority of cytotoxic amphipathic peptide molecules are cationic and they do exhibit selective toxicity against microbes. There are also hydrophobic or slightly anionic α-helical peptides which exhibits less selectivity towards microbes (Goodwin *et al*., 1996).
(b) Peptides consisting of β-sheets, Connected by Intermolecular Disulfide Bridges

A few of the known AMPs form a single β-hairpin structure and are approximately 20 residues long containing one or two disulfide linkages. B-sheet peptides are cyclic peptides constrained either by disulfide bond or cyclization of the peptide backbone. They largely exist in the β-sheet conformation in aqueous solution that may be further stabilized upon interaction with lipid surface. Defensins are the most characterized β-sheets forming antimicrobial peptides. Different mechanisms involving either the perturbation of lipid bilayers or the formation of discrete channels have been suggested for these peptides (Tamumura et al., 1998).

(c) Peptides with Extended Structures, Characterized by Over Representation of One or More Amino Acids

Some AMPs are composed of high numbers of regular amino acids. The structural conformations of such peptides are different from the regular α-helical or β-sheet peptides. Histatin, a peptide isolated from human saliva is rich in histidine residues and is active against Candida albicans (Xu et al., 1991).

(d) Peptides Composed of Rare and Modified Amino Acids

Few peptides are unusual as they are composed of rare modified amino acids. Best examples of such peptides are those produced by the bacteria themselves. Nisin, a lantibiotic, is one such peptide produced by Lactococcus lactis and is composed of rare amino acids like lanthionine, 3-methyllanthionine, dehydroalanine and dehydrobutyrine. The peptide is active against gram-positive bacteria and shows no defined structural conformation in water, while it reveals several β-turn structures when bound to dodecylphosphocholine (Van Den Hooven et al., 1996). Another peptide leucocin A, a 37-residue AMP isolated from Leuconostoc gelidum is shown to form an amphiphilic conformation well suited for interacting with membranes. Such peptides undergo post-translational modification that result in conformations not seen in other classes of antimicrobial peptides (Gibbs et al., 1998). With short size, easy to synthesize and being proteolytically stable, this class of peptides holds considerable potential in fighting against emerging infectious disease.
AMPs have 50% hydrophobic aminoacid residues and a low proportion of both neutral polar and negatively charged aminoacids (Hancock and Chapple, 1999). AMPs including both cationic and neutral peptides are secreted from both gram negative and gram positive bacteria. Nisin was the first discovered bacteria and derived cationic antimicrobial peptide. AMPs have a property of target specificity and selective toxicity. These properties are due to difference in membrane composition, hydrophobicity and charge of the mammalian cells and prokaryotic cells.

The prokaryotic cells are negatively charged due to the presence of phosphatidyl glycerol (PG), cardiolipin (CL) and phosphatidyl serine (PS). The mammalian cytoplasmic membranes are neutral in charge due to zwitter ionic phospholipids like phosphatidyl ethanolamine (PE), phosphatidyl choline (PC) or sphingomyelin. The cationicity of AMPs promote interaction with prokaryotes. Variety of fundamental differences exist between malignant cells and normal cells that likely account for the ability of certain AMPs to kill cancer cells (Hoskin and Ramamoorthy, 2008)

Antimicrobial peptides form structures with a positively charged face as well as a hydrophobic face, there are also some hydrophobic interactions between the hydrophobic regions of the antimicrobial peptides and the zwitter ionic phospholipids (electrically neutral) surface of the bacterial membranes, which act only as a minor effect.

Most cytotoxic peptides are positively charged due to the presence of lysine and arginine residues in their sequences. The net charge of the molecules ranges from +2 to +9 and can vary with pH as a result of the ionization state of various residues. A positive charge facilitates the binding of antimicrobial peptides to negatively charged membranes. When the positive activity become too high, the membrane activity of peptide may decrease because the strong electrostatic interactions anchor the peptide to the lipid head group region or because the repulsion between positively charged side chains, intra or inter molecular obstructs the formation of pores (Matsuzaki, 1999).

The mode of action of AMPs induces membrane defects such as phase separation, membrane thinning, pore formation, bilayer disruption depending on the molecular property of the peptide. Recently various mode of action other than membrane permeabilization have been
demonstrated which include inhibition of nucleic acid synthesis, protein synthesis, enzymatic activity and cell wall synthesis (Brogden, 2005).

Antimicrobial peptides are largely able to exhibit their activity because of their amphipathic or amphilic nature and because of the presence of regions with folded structures having positively charged residues (Oren et al., 1997). Different peptides may be membrane permeabilizing at their minimal effective concentration or at concentration well above or well below these concentrations (Powers et al., 2004).

**Mode of Action in Gram Negative Bacteria**

The mechanism of action on gram-negative organisms, which is the best studied, involves the initial displacement of Mg\(^{2+}\) and Ca\(^{2+}\) cations that stabilize lipopolysaccharides. This mediates the formation of perturbed areas, through which the peptide translocates the outer membrane by a process termed self promoted uptake (Zhang et al., 2000). The peptide then associates with the outer monolayer of the cytoplasmic membrane. It is at this point that membrane disruptive and non-membrane-disruptive mechanisms diverge, depending on whether this reorientation leads to perturbation of integrity of the cytoplasmic membrane or to peptide translocation into the cytoplasm, targeting other cell components such as cytoplasmic anionic molecules like DNA or enzymes (Dufrene and Müller, 2005).

**Mode of Action in Gram Positive Bacteria**

The mechanism of action on gram-positive organisms involves the interaction of antimicrobial peptide with the peptidoglycan layer. For Gram-positive cells, exposure to antimicrobial peptides results in immediate increase in water and ion flow, an efflux of K\(^+\) ions, swelling and osmotic imbalance (Matsuzaki, 1999)

Various mechanisms were proposed to describe the membrane permeabilizing action of AMPs, they are

1. Barrel stave mechanism
2. Carpet model mechanism
3. Torroidal pore mechanism
Barrel Stave Mechanism

The barrel stave mechanism describes the formation of transmembrane channel/pores by bundles of amphipathic α helices, such that their hydrophobic surfaces interact with the lipid core of the membrane and their hydrophilic surfaces point inward producing an aqueous pore (Matsuzaki, 1998). The trans membrane pore formation involves binding of peptide monomers to the membrane in a helical fashion followed by insertion of the helices into the hydrophobic core of the membrane. Progressive recruitment of additional monomers increases the pore size leading to leakage of cell contents and thereby death of the cell. Pore formation accompanies the reorientation of the helix from the parallel state to the perpendicular membrane spanning state.

Carpet Model Mechanism

In this model, the peptides at high concentration are in contact with the phospholipid head group on the outer leaflet of the membrane and cause membrane permeation. The peptides first bind to the surface of the target membrane and cover it in a carpet like manner. In this model, the peptides are not inserted into the hydrophobic core of the membrane nor do they assemble with their hydrophilic surfaces facing each other (Reddy et al, 2004). The following steps are involved in this model.

- Preferential binding of the peptide monomers to the phospholipid head groups. This is followed by the alignment of peptide monomers on the surface of the membrane such that their hydrophilic surface is facing the phospholipid head groups or water molecules leading to the reorientation of the hydrophilic residues towards the hydrophobic core of the membrane. Then the peptide disintegrates the membrane by disrupting the bilayer curvature (Matsuzaki, 1998). The carpet model was proposed for the first time to describe the mode of action of dermaseptin (Pouny et al., 1992).

Torroidal pore mechanism

Other mechanism of membrane permeabilizing includes torroidal pore formation. In this model the peptides are located closer to the head group region with an initial orientation parallel to the lipid bilayer surface. In this orientation the hydrophilic side of the helix is exposed to the hydrophilic lipid head groups and the water phase outside bilayers, while the hydrophobic face of the helix is buried in the hydrophobic core of the membrane to minimize the net free energy of
folding process. Aggregation of peptides to a sufficient local concentration increases the curvature strain on the membrane surface to extent that torroidal pores form.

AMPs have variety of activities like antibacterial, antiviral, antifungal, anticancer and antiparasitic activities. So they are being developed as a new source of antibiotics (Hancock and Chapple, 1999).

**Other Modes of Action of Antimicrobial Peptides**

Apart from permeabilizing membrane, disrupting the membrane integreity and then ion channel formation, AMPs also have other mechanisms for killing pathogens. Other modes of action are the stimulation of host-defense mechanism and then receptor mediated signaling activities of some peptides. Many AMPs actually do act by entering the selected cells and binding to some intracellular target such as DNA, interfering with their metabolite function. Another emerging view is that many peptides could act synergistically with other host molecules with antimicrobial activity to kill microbes. Thus, the importance of AMPs extends beyond their antimicrobial activities, such as their broad biological activities indicate they are effectors, providing communication between innate and adaptive immune systems (Yang et al., 2002).

**Therapeutic Considerations of AMP**

The therapeutic potential of AMP is attributed to their membrane lytic properties. The peptides have demonstrated their ability to kill rapidly a broad spectrum of microorganisms including multidrug resistant bacteria, fungi and viruses. They also combat other pathogens including protozoa. The following comments highlight emerging concepts of AMP as a therapeutic potential (Michael et al., 2003). They are

A. Reconstitution of conventional antibiotic efficacy

B. Unique and specific microbial targets

C. Targeting strategic microbial response pathways

D. Engineering new anti-infective based on peptide structure and function
A. Reconstitution of Conventional Antibiotic Efficacy

The most obvious potential therapeutic applications for antimicrobial peptides or derived mimetics relate to their use to reconstitute or amplify the antimicrobial efficacies of conventional antibiotics. For example, given their propensity to permeabilize target microbial membranes, antimicrobial peptides may facilitate conventional agents in overcoming access-based resistant mechanisms such as reduced uptake or enhanced efflux. Alternatively, peptides that interact with intracellular processes or targets could be engineered or selected to noncompetitively augment the targets and mechanisms of classical antibiotics (Tang et al., 2002).

B. Unique and Specific Microbial Targets

Structural and functional attributes unique to antimicrobial peptide interactions with pathogens offer new insights for development of novel anti-infective agents derived from these ancient host defense molecules. Molecular determinants that are emerging as potential targets for antimicrobial peptide strategies include microbial receptors, metabolic processes, energetics or essential pathways, virulence factors such as surface adhesins and envelope proteins, as well as intracellular targets such as ribosomes, mitochondria, or nucleic acids. Exploiting these developments will require further dissection of the molecular basis underlying peptide differentiation of appropriate microbial targets from those of hosts, emphasizing selective activity without concomitant host cytotoxicity (Cole et al., 2001).

C. Targeting Strategic Microbial Response Pathways

It is likely that antimicrobial peptides target constitutive and inducible properties of pathogens as targets of their mechanisms of action. For example, modification of characteristic membrane energetics, surface ligands, or expression of virulence factors may be avenues exploited by antimicrobial peptides in host defense. The unregulated activation of signal transduction pathways or response regulons upon exposure to antimicrobial peptides or their analogs may lead to pathogen incapacitation and eventual cell death due to global misregulation. Even if non lethal, these effects may render pathogens at greatly increased vulnerability to clearance by other host defense mechanisms (Yeaman et al., 2002).
D. Engineering New Anti Infective Based on Peptide Structure and Function

Advances in understanding the structural and mechanistic aspects of antimicrobial peptides may accelerate the development of improved anti infective agents. For example, a clearer recognition of how antimicrobial peptides differentiate between pathogen and host cells holds the promise of designing agents with greater selective toxicity. In this respect, efforts to identify and constrain peptides to antimicrobial conformations may allow the engineering of novel agents with potent efficacy against even the most antibiotic-resistant pathogens, without concomitant host cytotoxicity (Shankaramma et al., 2002). Thus, antimicrobial peptide structure and function as conserved by nature over an evolutionary time span offers hope for discovery and development of improved agents to prevent or treat infectious diseases caused by pathogens that resist conventional antimicrobial agents.

Advantages of Antimicrobial Peptides over Conventional Antibiotics

1. Unusually broad spectrum of activity.
2. Kill multi drug resistant bacteria at similar concentration.
3. Compared with conventional antibiotics, the killing of bacteria by peptides is extremely rapid and can involve multiple cellular targets (Brogden, 2005).
4. They are able to neutralize bacterial endo toxins and prevent development of sepsis.
5. Another advantage for the host of investing AMP as defense weapons is that given the non-specificity of their mechanism. It is not easy for microbial pathogens to develop resistant mutants to overcome peptide attack.
6. A few peptides have also been found to be cytotoxic to sperm, that have been explored to their contraceptive potential (Reddy et al., 2004).
7. Their diverse application is that they can be used as single antimicrobials, or in combination with other antibiotics for a synergistic effect, or as immunomodulatory and endo-toxin neutralizing compound (Zasloff, 2002).

The emergence of multidrug resistant bacterial strains throughout the globe limits the effectiveness of the current drugs and significantly limits the treatment leading to prolonged infections (Hancock, 2005). Cancer cells develop resistance that is mediated by the over expression of the multidrug resistance proteins that pump the drug out of the cells and thus render the drugs ineffective (Fez Tomas, 2006). There is an urgent need to develop new classes of anticancer drug with new mode of action that selectively target the cancer cells. Cationic
peptides are widely distributed in living organisms laying a variety of functions. They are often referred to as antibacterial or antimicrobial peptides due to their well characterized role in innate immunity against infectious agents (Hanlock and Scott, 2000).

With these backgrounds the present study has been designed for the isolation and characterization of antimicrobial protein from *Bacillus subtilis* and *Bacillus pumilus* for its antibacterial, antifungal and anticancer potentials.