Review of Literature

Pseudomonas aeruginosa is a Gram negative, aerobic, rod shaped bacterium with unipolar motility (Ryan et al., 2004). It is a common bacterium which can cause disease in animals and humans, found in soil, water, and most man-made environments throughout the world. It thrives not only in normal atmospheres, but also with little oxygen, and has thus colonized in many natural and artificial environments. Because it thrives on moist surfaces, this bacterium is also found on and in medical equipments including catheters, causing cross infections in hospitals and clinics. It uses a wide range of organic material for food; in animals the versatility enables the organism to infect damaged tissues or people with reduced immunity (Balcht et al., 1994).

Multidrug-resistant P. aeruginosa strain is defined as an isolate intermediate or resistant to atleast three drugs of the following classes: β-lactams (piperacillin, piperacillin-tazobactam, ceftazidime, cefepime, ticarcillin, ticarcillin-clavulanate), carbapenems (imipenem, meropenem), aminoglycosides (amikacin, gentamycin, tobramycin) and fluoroquinolones (ciprofloxacin). The introduction of carbapenems represented a great advance for the treatment of serious bacterial infections caused by beta-lactam resistant bacteria. Carbapenems are the drug of choice for multidrug-resistant P. aeruginosa and ESBL producing organisms. However, resistance to carbapenems due to reduced uptake of drug leads to imipenem/meropenem resistant isolates (Behera et al., 2008).

Biology of Pseudomonas aeruginosa

P. aeruginosa is a slender Gram negative bacillus, 1.5-3µm x 0.5µm, actively motile by a polar flagellum. Clinical isolates are often piliated. It is non-capsulated but many strains have a mucoid slime layer. Mucoid strains, particularly isolates from cystic fibrosis patients have an abundance of extracellular polysaccharides composed of alginate polymers. This forms a loose capsule in which micro colonies of the bacillus are enmeshed and protected from host defences (Ananthanarayanan et al., 2000).
Cultural Characteristics

*P. aeruginosa* is an obligate aerobe, but can grow anaerobically if nitrate is available. Growth occurs at a wide range of temperatures, 6-42°C, and the optimum being 37°C. It grows well on ordinary media, producing large, opaque, irregular colonies, with a distinctive, musty, mawkish or earthy smell. Iridescent patches with a metallic sheen are seen in cultures on nutrient agar. It grows on MacConkey and DCA media, forming non-lactose fermenting colonies. Many strains are haemolytic on blood agar. In broth it forms a dense turbidity with a surface pellicle. Most but not all *P. aeruginosa* cultures have a characteristic fruity odour due to the production of O-aminoacetophenone from tryptophan (Pitt, 1996).

Pigment Production

The production of a blue-green pigment which diffuses into the surrounding medium confirms the identity of *P. aeruginosa*, but many cultures do not form pyocyanin except on special media and some do not form it at all. The ability to form pyocyanin may be irreversibly lost in culture (Pitt, 1996). Four different pigments have been described in *P. aeruginosa*: pyocyanin, fluorescein, pyorubrin and pyomelanin. Pyocyanin is a water soluble blue-green phenazine pigment produced by active cultures of *P. aeruginosa*. Pyocyanin has antibiotic activity against bacteria, fungi and protozoa, but is of little therapeutic value because it is quite toxic to eukaryotic cells (Baron *et al*., 1981).

Fluorescein (pyoverdin) is insoluble in chloroform but soluble in water. It imparts a yellowish tinge to cultures but this is sometimes not easy to detect unless cultures are examined under ultraviolet light. Fluorescein has powerful siderophore activity (Meyeret *et al*., 1978) the expression of which is increased by iron limitation. Strains of *P. aeruginosa* that elaborate a bright red water soluble pigment were described initially by Gessard. Meader *et al*., (1925), who designated it pyorubrin and found it to be characteristic of fresh isolates. Pyorubrin is irreversibly reduced to a colourless form in reduced oxygen concentration. It is a phenazine pigment that is
Insoluble in chloroform. The production of pyomelanin, a brown to black pigment, is uncommon (Yabuuchi et al., 1972), less than 1% of strains form it (Phillips, 1969).

**Isolation and Characterization of *Pseudomonas aeruginosa***

The metabolism is oxidative and non-fermentative. Glucose is utilized oxidatively, forming acid only. Indole, Methyl red, Voges Proskauer and Hydrogen sulfide tests are negative. Nitrates are reduced to nitrites and further to gaseous nitrogen. Catalase, oxidase and arginine hydrolase tests are positive (Ananthanarayan et al., 2000). It oxidizes gluconate and produces slime (Haynes, 1951), reduces tetrazolium salts to form red colonies (Selenka, 1958), reduces selenite (Lapage et al., 1968) and deaminates acetamide (Buhlmann et al., 1961). Of the fluorescent pseudomonads, only *P. aeruginosa* will grow in mineral salts medium with geraniol. *P. aeruginosa* forms hydrocyanic acid both *in vivo* and *in vitro* (Patty, 1921).

Selective media for isolation of *P. aeruginosa* include nutrient agar supplemented with antibiotics (Wong et al., 1983). Cetrimide agar inhibits Gram positive organisms and Pseudomonas isolation agar is the usual selective media used for isolation (Lambe et al., 1972; Fonscea et al., 1986). GMAC (Glycerol, Mannitol, Acetamide and Cetrimide) agar is used routinely with nitrofurantoin broth, with respect to productivity and selectivity (Thom et al., 1971). Yeast extract media is used for mucoid strain isolation (Allison et al., 1987).

**Prevalence and Pathogenesis***

Occasionally, *P. aeruginosa* can colonize human body sites, with a preference for moist areas, such as the perineum, axilla, ear, nasal mucosa and throat; as well as stools. The prevalence of colonization by *P. aeruginosa* in healthy subjects is usually low, but higher colonization rates can be encountered following hospitalization, especially amongst subjects treated with broad-spectrum antimicrobial agents. Colonization is common in the respiratory tract of mechanically ventilated patients, in the gastrointestinal tract of patients receiving anticancer chemotherapy, and on the skin of burn patients (Morrison et al., 1984; Pollack, 2000).
P. aeruginosa is typically an opportunistic pathogen that seldom causes disease in healthy subjects. Normally, for an infection to occur, some disruption of the physical barriers (skin or mucous membranes), or by-passing of them (e.g., by urinary catheters, endotracheal tubes or other invasive devices), and/or an underlying dysfunction of the immune defence mechanisms, such as neutropenia, is necessary. As a consequence, P. aeruginosa is mostly a nosocomial pathogen. According to data from the Centre for Disease Control and Prevention National Nosocomial Infection Surveillance System, in the USA, P. aeruginosa was the second most common cause of nosocomial pneumonia, the third most common cause of nosocomial urinary tract infections, and the seventh most common cause of nosocomial bacteraemia (NNIS, 1999). In Europe, P. aeruginosa was found to be the third most common isolate from nosocomial infections in intensive care units (ICUs) (Vincent et al., 1995).

Nosocomial infections caused by P. aeruginosa most frequently involve the respiratory tract, the urinary tract and wounds. P. aeruginosa is amongst the leading causes of nosocomial pneumonia, especially in mechanically ventilated patients. Mortality rates ranging from 40% to more than 60% have been reported in bacteremic nosocomial pneumonia and in ventilator associated pneumonia (Crouch Brewer et al., 1996; Mayhall, 1997; Rello et al., 1997).

Nosocomial urinary tract infections caused by P. aeruginosa are usually related to catheterization or other invasive procedures, and may be complicated by bacteraemia (Pollack, 2000). Wound infections are particularly serious in burn patients, where they are often complicated by bacteraemia (Mousa, 1997). P. aeruginosa bacteraemia and septic shock are primarily observed in immuno-compromised patients, and are associated with high mortality rates (from one-third to almost two-thirds of cases) (Bodey et al., 1985; Gallagher et al., 1989; Siegman - Igra et al., 1998; Collin et al., 2001).

All situations associated with severe neutropenia and mucosal ulcerations, such as haematological malignancies, cancer chemotherapy and organ transplantation, create a significant risk for the development of P. aeruginosa bacteraemia (Pollack, 2000;
Oll et al., 1993; Aquino et al., 1995; Fishman et al., 1998; Pizzo, 1999). Other predisposing factors include diabetes mellitus, immunoglobulin deficiency states, severe burns, steroid therapy, surgery and the use of invasive devices (Pollack, 2000). In cancer patients, *P. aeruginosa* can be responsible for up to 30% of culture-proven cases of bacteraemia, with mortality rates ranging from 5% to 50% (Maschmeyer et al., 2000).

*P. aeruginosa* typically infects the pulmonary tract, urinary tract, burns, wounds, and also causes other blood infections in immuno-compromised hosts. It's the most common cause of burn and external ear infections, and is the most frequent colonizer of medical devices such as catheters. *Pseudomonas* can in rare circumstances cause community acquired pneumonias, (Fine et al., 1996) as well as ventilator-associated pneumonias. One in ten hospital-acquired infections is from *Pseudomonas*. Cystic fibrosis patients are also predisposed to *P. aeruginosa* infection of the lungs.

*P. aeruginosa* is a major pathogen in cystic fibrosis patients. The abnormal airway epithelia of these patients allow long-term colonization by *P. aeruginosa* and, once infected, they rarely, if ever, clear the microorganism, which, in turn, plays a critical role in the progression of lung disease (Pollack, 2000 and Davies, 2002). Finally, *P. aeruginosa* can be an important cause of morbidity and mortality in both paediatric and adult patients with acquired immuno-deficiency syndrome with very low CD4 counts (Dropulic et al., 1995; Vidal et al., 1999; Manfredi et al., 2000).

**Virulence Factors and Enzymes**

**Virulence factors**

The virulence mechanisms of *P. aeruginosa* are complex and only partially understood. Adherence mediated by pili and other adhesions appears to be important for the colonization of mucous membranes and other surfaces (Prince, 1992). The production of a mucoid exo-polysaccharide matrix (lipopolysaccharide, LPS) that surrounds the cells and anchors them to each other and to the environment is important for growth as a biofilm, in which the bacterial cells are protected from the host innate
and immune defences and are overall less susceptible to antibiotics (Boyd et al., 1995; Drenkard et al., 2002).

**Pyocyanin**

Pyocyanin pigment is probably a determinant of virulence. It has been shown to act as an inhibitor of mitochondrial enzymes in mammalian tissue (Armstrong et al., 1971) and to cause disruption and cessation of ciliary beat on ciliated nasal epithelium. This may be of significance for the ability of the organism to avoid clearance from respiratory mucosa by primary host defences (Wilson et al., 1987).

*Pseudomonas aeruginosa* is the only Gram negative bacillus capable of producing the very distinctive water-soluble pigment pyocyanin (Alberto Pichardo Reyes et al., 1981). Chemically, pyocyanin is 5-methyl-1-hydroxyphenazine and can undergo complex series of oxidation-reduction reaction (Grace Terranova et al., 1989; Stewart-tull et al., 1972). Pyocyanin is blue redox-active secondary metabolite, is a member a large family of tricyclic compounds known as Phenazine's. They are secreted at the late stationary phase and provide a characteristic blue colour to the medium (Fank et al., 1959).

**Genetics of Pyocyanin**

Mutational and biochemical analyses have identified two groups of gene products required for PCN biosynthesis. First, the MvfR transcription factor is required to activate the phnA-B genes (Mahajan-Miklos et al., 2001; Cao et al., 2001; Gallagher et al., 2002). The phnA-B gene products synthesize quinolone that regulate the phzRABCDEFG operons 1 and 2, the structural genes for phenazine synthesis (Mavrodi et al., 2002).

**Effect of Pyocyanin on Microbes**

It was theorized that the reducible nature of pyocyanin was important to the respiration of *Pseudomonas aeruginosa* because addition of pyocyanin increased the oxygen uptake of *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Staphylococcus aureus* and erythrocytes. This theory stated that pyocyanin could
function as an extracellular respiratory pigment. It was a particularly attractive theory for aerobic bacteria grown under conditions of low aeration in which the pyocyanin could be reduced, would be freely diffusible to participate in autooxidation by oxygen in more aerobic environments, and could recycle in subsequent reductive steps in bacterial respiration (Friedheim et al., 1931). It was found that pyocyanin actually inhibited the oxygen uptake of Vibrio cholerae and S. aureus (Schoental et al., 1941). The inhibitory nature of pyocyanin suggested an antibacterial activity for the pigment. A possible explanation for the respiration-linked antibiotic activity of pyocyanin was offered by the finding of superoxide radical generation during the autooxidation of reduced pyocyanin. In addition to this activity, pyocyanin also inhibited keto-acid oxidation in Pseudomonas fluorescens and Proteus vulgaris, but not in Escherichia coli (Hassan et al., 1980).

**Enzymes**

A number of extracellular products secreted by *P. aeruginosa*, including elastase, alkaline protease, cytotoxin, phospholipase C and rhamnolipid are also involved in the pathogenesis.

**Proteases**

*P. aeruginosa* produces several proteolytic enzymes which degrade a wide range of substrates including casein, elastin, gelatine, collagen and fibrin. At least three distinct proteases have been characterized a general protease, an alkaline protease and an elastase (Morihara, 1964), distinguished by their pH optima, substrate specificity and physical properties (Nicas et al., 1986). Alkaline protease requires calcium or cobalt for maximum activity and is inhibited by chelators. Elastase, metallo-enzyme containing zinc, is also inhibited by chelators, heavy metals and reducing agents.

Alkaline protease and elastase are regulated independently; elastase differs from alkaline protease in being formed in greater amount on synthetic than on complex media (Jensen et al., 1980) and in being more active against casein and immunoglobulin G. Excess iron tends to decrease elastase production.
Haemolysins

*P. aeruginosa* produces 2 distinct haemolysins, a heat-labile enzyme, phospholipase C (Esselman *et al.*, 1961) and a heat-stable rhamnolipid (Sierra, 1960). Phospho-lipase C hydrolyzes phosphatidylcholine in erythrocyte membrane to phosphorylcholine and diacylglycerol and probably acts synergically with an alkaline phosphatase to cleave inorganic phosphate from phospholipids. Phospholipase C is not produced in media containing high levels of inorganic phosphate; its action gives rise to opacity around the growth on egg-yolk agar (Esselman *et al.*, 1961). Most strains are lipolytic and will degrade a wide variety of fats and tweens 20 and 80. Extracellular lipase is excreted by *P. aeruginosa* during the late logarithmic growth phase and appears to be tightly bound to lipopolysaccharide (Steur *et al.*, 1986).

Exotoxins

Exotoxin A is produced by most *P. aeruginosa* strains that cause clinical infections. Exotoxin A catalyzes ADP ribosylation and inactivation of elongation factor 2, leading to inhibition of protein biosynthesis and cell death (Wick *et al.*, 1990). Exotoxin A is responsible for local tissue damage, bacterial invasion and possibly immunosuppression (Vidal *et al.*, 1993).

Anti-pseudomonal Drugs

The most important anti-pseudomonal agents include some β-lactams (ticarcillin, ureidopenicillins, piperacillin, cefoperazone, ceftazidime, cefepime, aztreonam, imipenem and meropenem), aminoglycosides (gentamicin, tobramycin, netilmicin and amikacin) and fluoroquinolones (of which ciprofloxacin remains the most active compound) (Giamarellou *et al.*, 2001; Jones *et al.*, 2001). Polymyxins (polymyxin B and colistin) are also active but, due to their higher toxicity, are usually considered only for multidrug-resistant (MDR) strains that are resistant to the other agents (Giamarellou, 2002).

The β-lactam antibiotics act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. Penicillin-binding proteins (PBPs) are involved in the final transpeptidation step in the synthesis of the peptidoglycan. The β-lactam antibiotics
bind to the active site of PBP’s, prevented the transpeptidation and disrupts cell wall synthesis.

Aminoglycosides are a vital component of anti-pseudomonal chemotherapy implicated in the treatment of a variety of infections (Bartlett, 2003; Gilbert et al., 2003). These agents are bactericidal and exhibit synergy with other antimicrobials, most notably β-lactams, with which they are often, administered for the treatment of P. aeruginosa infections. Aminoglycosides inhibit protein synthesis by binding to the 30S subunit of bacterial ribosome. Fluroquinolones act by inhibiting replicative enzymes (DNA gyrase), there by affecting DNA replication.

Drug Resistance

With the widespread use of extended spectrum antibiotics, advancements in medical and surgical technology, P. aeruginosa has become resistant to a variety of antimicrobial agents (Livermore, 2002; Nordmann et al., 2002) such as β-lactams, amino glycosides, chloramphenicol, quinolones, tetracyclines and sulphonamides.

Multidrug-resistance of P. aeruginosa is a complex and multifactorial problem over the past few decades (Borso, 1989) both in terms of its origin (Bryan, 1979) and developing resistance during treatment (Bonfiglio et al., 1998). β-lactams are the most widely used antimicrobials worldwide, in the developed countries they account for 60-70% of all antimicrobials prescribed, both in hospitals and in the community (Standing Medical Advisory Committee, 1998), and resistance to β-lactam antibiotics is due to synergistic action of low outer membrane permeability and high β-lactamase activity (Nikaido et al., 1987). Resistance to aminoglycosides and fluoroquinolones has also become common throughout the world.

Mechanisms of Antibiotic Resistance

The most common mechanisms of P. aeruginosa resistance to anti-pseudomonal drugs have been the alteration of target site, production of β-lactamases; intrinsic drug resistance (which includes low membrane permeability and an active efflux system) and acquired resistance.
Alteration of Target Site

Chemical modifications in the antibiotic target may result in reduced affinity of the antibiotic to its binding site (Lambert, 2005). Resistance to β-lactams in P. aeruginosa is a result of modification in the active site of penicillin-binding proteins. The main mechanism in the development of resistance to fluoroquinolones is decrease in binding of the quinolones to enzymes because of changes in DNA gyrase enzyme and/or the topoisomerase enzyme. As the nucleotide and amino acid sequence of gyr A, gyr B, par C and par E genes needed for the synthesis of DNA topoisomerase are very similar to those of DNA gyrase enzyme, mutations occur in gyr B, par C genes and the resistance observed in P. aeruginosa is usually active against all quinolones (Algun et al., 2004).

Production of β-Lactamases

β-lactamases are enzymes that confer significant antibiotic resistance to their bacterial hosts by hydrolysis of the amide bond of the four-membered β-lactam ring (Wilke et al., 2005). The β-lactamases found in P. aeruginosa can belong to three different groups:

1. Narrow-spectrum active site-serine enzymes of molecular classes A and D (e.g., PSE-1, PSE-4 and some OXA-type enzymes) that efficiently degrade the anti-pseudomonal penicillins and cefoperazone, but have no significant activity against the other anti-pseudomonal cephems, monobactams or carbapenems (Livermore, 1995; Bush et al., 1995; Nass et al., 1999).

2. Extended-spectrum active site-serine enzymes of molecular classes A and D (e.g., PER-1, VEB-1, GES-1, GES-2, various OXA-type enzymes and, although rarely, also TEM- and SHV-type extended-spectrum variants) that, in addition to penicillins, can also degrade the anti-pseudomonal cephems and monobactams but not carbapenems (Livermore, 1995; Bush et al., 1995; Bradford et al., 2001; Mavroidi et al., 2001; Dubois et al., 2002)
3. Metallo-enzymes of molecular class B (eg. the enzymes of the IMP, VIM, SPM and GIM type) that efficiently degrade virtually all the anti-pseudomonal β-lactams except monobactams (Bush, 1998; Nordmann et al., 2002; Toleman et al., 2002; Docquier et al., 2003; Castanheira et al., 2004).

**Intrinsic Drug Resistance and Efflux Systems**

*P. aeruginosa* shows significant degrees of intrinsic resistance to a wide variety of antimicrobial agents including most β-lactams, chloramphenicol, tetracyclines and fluoroquinolones, due mainly to its low outer membrane permeability and to active efflux of antibiotics (Hancock, 1998). Several studies have shown that active efflux can be a mechanism of resistance for almost all antibiotics (Li et al., 1994a; Gill et al., 1999; Lin et al., 2002).

Multidrug-resistance efflux pumps are either chromosomally encoded or plasmid encoded and are ubiquitous proteins (Akama et al., 2005). They belong to five families of transporters namely; the major facilitator super-family (MFS), the adenosine triphosphate (ATP)-binding cassette (ABC) super-family, the small multidrug-resistance (SMR) family and the resistance nodulation-cell division (RND) super-family and the multidrug and toxic compound extrusion (MATE) family (Kumar and Schweizer, 2005). Four *P. aeruginosa* multidrug-efflux systems have been reported, all of which are members of the resistance-nodulation-cell division (RND) family (Westbrock-Wadman et al, 1999; Nikaido, 1998).

The RND family efflux pump, Mex AB-Opr M, of the opportunistic pathogen, *P. aeruginosa* has been extensively characterized. Like other tripartite efflux proteins, it consists of three membrane bound subunits, Mex A, Mex B, and Opr M, anchoring the inner and outer membranes. The Mex B subunit is central to the pump function, which spans the cytoplasmic membrane 12 times, it selects antibiotics to be exported, and is assumed to transport the substrates expending the energy of the proton gradient across the cytoplasmic membrane (Akama et al., 2004).
Resistance to β-lactam and non β-lactam antibiotics such as quinolones, tetracyclines, and trimethoprim has been attributed to efflux by the Mex AB-Opr M pump (Ziha-Zarifi et al., 1999). Other Mex efflux proteins namely Mex CD, Mex EF Mex XY mediating multidrug-resistance have also been cloned from the chromosome of *P. aeruginosa* (Mine et al., 1999).

### Acquired Antimicrobial Resistance

The acquired resistance to β-lactams can be due to horizontal transfer of β-lactamase genes and mutations. Resistance to anti-pseudomonal β-lactams is common and can result from one or more of several different mechanisms. The most probable one is mutations leading to the increased production of the AmpC β-lactamase and decreased amount of the Opr D porin protein. Loss of Opr D production can be due to deletions, substitutions or insertions that cause inactivation of the opr D gene (Yoneyama et al., 1993; Pirnay et al., 2002), but Opr D production can also decrease following regulatory mutations that cause both the down-regulation of Opr D and the up-regulation of the Mex E–Mex F–Opr N efflux system (Kohler et al., 1997).

Acquired resistance to aminoglycosides can be due to the production of aminoglycoside-modifying enzymes encoded by horizontally acquired resistance determinants, or by mutations that reduce aminoglycoside accumulation in the bacterial cell (Hancock et al., 1998). The most prevalent aminoglycoside modifying enzymes found in *P. aeruginosa* are the acetyl-transferases AAC-II (resistance to gentamicin, tobramycin and netilmicin), AAC (3)-I (resistance to gentamicin), AAC (3)-II (resistance to gentamicin, tobramycin and netilmicin) and AAC (6)-I (resistance to tobramycin, netilmicin and amikacin) and the adenyl-transferase ANT (2)-I (resistance to gentamicin and tobramycin) (Shaw et al., 1993; Miller et al., 1997).

Reduced aminoglycoside uptake could be due to mutations causing lipo-polysaccharide changes (Bryan et al., 1984) or up-regulation of efflux systems based on the Mex X-Mex Y linker-pump module (Masuda et al., 2000; Jo et al., 2003). Acquired resistance to fluoroquinolones can be due either to mutations that cause the
up-regulation of efflux systems, including Mex A-Mex B-Opr M, Mex C-Mex D-Opr J, Mex E-Mex F-Opr N and Mex X-Mex Y-Opr M (Poole, 2001), or to mutations of the topoisomerase targets (gyr A and also par C) (Nakano et al., 1997; Akasaka et al., 2001).

Finally, acquired resistance to polymyxins has been occasionally described in *P. aeruginosa* isolates from cystic fibrosis patients treated for long periods with the nebulised drug, and seems to be related to mutations causing changes in the outer membrane structure (Livermore, 2002). The genes encoding these enzymes may be inherently present on the bacterial chromosome or may be acquired via plasmid transfer and β-lactamase gene expression may be induced by exposure to β-lactams. The presence of β-lactamase gene in plasmid or chromosome can be identified by PCR amplification using specific primers.

**Plasmid Curing**

The presence of β-lactamase genes in plasmid can be removed or deleted by a process of plasmid curing. In general, physical loss of the plasmid is best demonstrated by its absence using either density centrifugation of cleared lysates of the strain in cesium chloride-ethidium bromide gradients (Clewell and Helinski, 1969) or the more rapid procedures involving the analysis of partially purified plasmid DNA on agarose gels (Birnboim and Doly, 1979; Klein et al., 1980). Agents that have been involved in isolating plasmid free cells include, Intercalating dyes (Hirota, 1960; Bouanchand et al., 1969); Coumermycin (Danilevskaya and Gregerov, 1980), SDS (Tomoeda et al., 1968, Salisbury et al., 1972).

**Intercalating Dyes**

Acriflavin, acridine orange, quinacrine and ethidium bromide have been used. Their mechanism of action seems to be a preferential inhibition of plasmid replication (Hohn and Korn, 1969). In this method the overnight cultures are inoculated at a concentration of about $10^2$-$10^4$ organisms per ml, in a series of tubes containing nutrient broth pH 7.6 and various concentrations of the curing agent. The optimal
concentration of the curing agent depends both on the bacterial strain and the agent used. After overnight growth at 37°C, the highest concentration of curing agent that still allows visible growth should be plated on nutrient agar plates and isolated colonies are tested for the loss of the phenotype of interest.

Plumbagin as Curing Agent

Plumbagin (5-hydroxy-2-methyl-1, 4-naphthaquinone), a compound derived from the root of the *Plumbago zeylanica* plant, was effective in selectively eliminating stringent, conjugative, multidrug-resistant plasmids from *Escherichia coli* strains. Simultaneous loss of resistance to antibiotics in plumbagin-treated cells indicated loss of plasmid. However, such R plasmids are refractory to treatment with acridine orange and sodium dodecyl sulphate, which are widely used in curing techniques (Lakhmi *et al*., 2005). Plumbagin is yellow pigment. It has been shown to have antimicrobial activity. In animals, it has antimalarial, anticarcinogenic, cardiotonic, antifertility action, and anti-therosclerosis effect (Didry *et al*., 1994).

Restriction Mapping

Many plasmids have been isolated from *Pseudomonas* species almost all of which are responsible for drug-resistance (drug resistant plasmids), mating (sex factor plasmids) or biodegradation of organomolecular compounds (degradative plasmids) (Charkrabarty, 1976). The plasmid has restriction sites for BamH1 and EcoRI (Sujoy Saha *et al*., 2000). The restricted sites are then cloned into plasmid vectors like pBR322 and propagated in *Escherichia coli*.

Carbapenems

Carbapenems are the drug of choice for multidrug-resistant *P. aeruginosa*. Carbapenems are the most potent β-lactams against *P. aeruginosa* because of their strong affinity to penicillin binding proteins, stability against most serine β-lactamases and high permeability across the outer membrane (Livermore, 1995, 2001). The carbapenems available for use in India are imipenem and meropenem. However, intensive use of carbapenems has facilitated the emergence of carbapenem-resistant
**Review of Literature**

*P. aeruginosa*. Resistance to carbapenems in *P. aeruginosa* is often due to impermeability, which arises via the loss of the opr D porin (Livermore, 1992), the up regulation of an active efflux- pump system present in the cytoplasmic membrane of these organisms (Kohler et al., 1999), or the production of carbapenem hydrolyzing enzymes-carbapenemases (Livermore et al., 2000).

Outer membrane proteins (OMPs) are of particular interest in *P. aeruginosa* due to their cell-surface exposure and their involvement in transport of antibiotics. Three large paralogous families were identified, the Opr D family of specific porins (19 genes), the Ton B-family of gated porins, which includes proteins involved in iron siderophore uptake (34 genes), and the Opr M family of outer membrane proteins involved in efflux or secretion (18 genes). Most of the carbapenem resistant *P. aeruginosa* lack the outer membrane porin protein D2 (Buscher et al., 1987; Quinn et al., 1986; Tries et al., 1990), which forms a specific channel for basic amino acids and carbapenems (Gotoh et al., 1990; Satake et al., 1990; Trias et al., 1990).

Low level resistance to imipenem (MIC 8-32µg / ml) in *P. aeruginosa* is mostly due to reduced uptake of the drug as a result of loss of the porin opr D (Livermore, 1995). Resistance to meropenem may also arise via over expression of the Mex A-Mex B-opr M efflux pump (Poole, 2001). The carbapenemases are class B metallo-β-lactamases (IMP, VIM) or class D-oxacillinases (OXA 23 to OXA 27) or class A-clavulanic acid inhibitory enzymes (SME, NMC, IMI, KPC). High level resistance to carbapenems (MIC >32µg/ml) can be caused by the production of metallo-β-lactamases.

**Metallo-β-Lactamases**

Metallo-beta-lactamase (MBL) belongs to a group of β-lactamase which requires divalent cations of zinc as cofactors for enzyme activity. These have potent hydrolyzing activity not only against carbapenem but also against other β-lactam antibiotics (Bush, 1998). *Pseudomonas aeruginosa* producing metallo-β-lactamases (MBLs) was first reported from Japan in 1991 (Watanabe et al., 1991) and since then
has been described from various parts of the world, including Asia (Lee et al., 2004; Yan et al., 2001; Yatsuyanagi, 2004), Europe (Lagatolla et al., 2004) South America (Gales et al., 2003), and North America (Toleman et al., 2004).

In laboratory based surveillance in Japan conducted during 1992-2002, MBL producing P. aeruginosa were identified at a rate of 0.4-1.9% (Hirakata et al., 1998; Kimura et al., 2005; Nishio et al., 2004; Yamasaki et al., 2003). In 2002 from India, Navneeth et al., first reported MBL production in P. aeruginosa to be 12%. Since then, the incidence of MBL production in P. aeruginosa has been reported to be 10-30% from various clinical specimens across the country (Taneja et al., 2003). MBL producing isolates have also been responsible for serious infections, such as septicaemia and pneumonia (Cornaglia et al., 2000), and have been associated with failure of therapy with carbapenems (Nordmann et al., 2002).

The genes responsible for the production of MBLs are typically part of an integron structure and are carried on transferable plasmids but can also be part of the chromosome (Poirel et al., 2002). Therefore, because of the integron-associated gene cassettes, P. aeruginosa isolates producing MBLs are often resistant to different groups of antimicrobial agents, which can be transferred to various types of bacteria (Nordmann et al., 2002). The appearance of MBL genes and their spread among bacterial pathogens is a matter of concern with regard to the future of antimicrobial chemotherapy.

MBLs can be divided into four categories according to their molecular structures, namely, the IMP, VIM, GIM, and SPM types (Castanheira et al., 2004; Lauretti et al., 1999; Osano et al., 1994; Toleman et al., 2002). The IMP and VIM are the most common types of Metallo-beta-lactamases (Senda et al., 1996). Since 1991, when Japan first reported plasmid-mediated IMP-1-type MBL in P. aeruginosa, European countries have followed up with reports of VIM-1 and VIM-2 types of MBL.

IMP type enzymes were the first acquired MBL to be detected in clinical isolates of Enterobacteriaceae, P. aeruginosa, and other non fastidious Gram negative
non-fermenters. IMP enzyme (imipenamases) is one type of broad spectrum MBL. Several variants of IMP enzymes may diverge from each other by single or several aminoacid substitutions. To date 21 subtypes of IMP and 11 of VIM have been identified. New subtypes of IMP and VIM are constantly appearing (Lagatolla et al., 2004). Second dominant group of MBL is the VIM (Verona integron-encoded enzyme) type, Veronese imipenemase VIM1 was first described first in Verona, Italy from a *P. aeruginosa* isolate (Lauretti et al., 1999).

The prevalence of different MBL genotypes varies in different countries and regions (Lagatolla et al., 2004; Poirel et al., 2000). In Asian countries and regions, two genotypes, IMP and VIM, are prevalent. The IMP is mainly found in Japan, while the VIM predominates in Korea, and Taiwan, China (Yan et al., 2001; Kim et al., 2005; Niitsuma et al., 2001; Yu et al., 2006).

Several phenotypic methods are available for the detection of MBL-producing bacteria. All these methods are based on the ability of metal chelators, such as EDTA and thiol-based compounds, to inhibit the activity of MBLs. These tests include the double-disk synergy tests using EDTA with imipenem (IPM) or ceftazidime (CAZ) (Lee et al., 2001, 2003; Yan et al., 2004), 2-mercaptpropionic acid with CAZ or IPM (Arakawa et al., 2000), the Hodge test (Lee et al., 2001, 2003), a combined disk test using EDTA with CAZ or IPM (Yan et al., 2004, Yong et al., 2002), the MBL Etest (Walsh et al., 2002), and a microdilution method using EDTA and 1,10-phenanthroline with IPM (Magliavacca et al., 2002). But 2-mercaptpropionic acid and 1,10-phenanthroline is toxic.

The IPM-EDTA disk method described by Yong et al. used 750 µg EDTA in combination with IPM disks with a zone difference of ≥7 mm between IPM alone and with EDTA (Yong et al., 2002). They reported excellent sensitivity and specificity to detect VIM-2 and IMI-1-producing *P. aeruginosa* and *Acinetobacter* spp. (Yong et al., 2002). A PCR detection assay was published in 1996 for the detection of Gram-negative bacteria producing IMP-1 (Senda et al., 1996), and a PCR typing scheme for
Infectious diseases are the leading cause of death world-wide. Antibiotic resistance has become a global concern (Westh et al., 2004). The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug-resistant pathogens (Bandow et al., 2003). With the discovery of antibiotics and their use as chemotherapeutic agents, there arose a belief in the medical fraternity that this would lead to the eradication of infectious diseases. However diseases and disease agents that were once thought to have been controlled by antibiotics are returning in new forms resistant to antibiotic therapies (Levy and Marshall, 2004). Incidents of epidemics due to such drug resistant microorganisms are now a common global problem posing enormous public health concern (Iwu et al., 1999).

**The Use of Resistance Modifying Agents**

Several studies have reported that antibiotic combinations can have synergistic benefits and interactions between existing antibiotics (Bayer et al., 1980; Hooton et al., 1984; Cottagnoud et al., 2000; Hallander et al., 1982). Taylor et al., (2002) suggested that the use of agents that do not kill pathogenic bacteria but modify them to produce a phenotype that is susceptible to the antibiotic could be an alternative approach to the treatment of infectious disease. Such agents could render the pathogen susceptible to a previously ineffective antibiotic, and because the modifying agent applies little or no direct selective pressure, this concept could slow down or prevent the emergence of resistant genotypes.

The inhibition of resistance expression approach was successfully used in the production of Augmentin, a combination of amoxycillin and clavulanic acid (Reading and Cole, 1977). In the case of *P. aeruginosa*, clavulanic acid is an inhibitor of class-A β-lactamases which is co-administered with amoxicillin. The combination has been used clinically since the late 1970s (Neu et al., 1993). A similar approach can be used for target-modifying enzymes and for efflux systems.
Herbal Remedies

Herbal remedies are viewed as a reemerging health aid in a number of countries (UNESCO, 1996). This can be traced to both the increasing costs of prescription drugs for the maintenance of personal health and the emergence of antibiotic-resistant strains in the case of infectious diseases. In the industrialized countries, the extraction and development of many drugs and chemotherapeutics from medicinal plants have been increasing (UNESCO, 1998). Plants have traditionally provided a source of hope for novel drug compounds, as plant herbal mixtures have made large contributions to human health and well-being (Iwu et al., 1999). Owing to their popular use as remedies for many infectious diseases, searches for substances with antimicrobial activity in plants are frequent (Betoni et al., 2006; Shibata et al., 2005).

Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found in vitro to have antimicrobial properties (Lewis and Ausubel, 2006; Cowan, 1999). Biologically active compounds from natural sources have always been a great interest for scientists working on infectious diseases (Perumalsamy et al., 2000). Many of these indigenous medicinal plants are used as spices and food plants. They are also sometimes added to foods meant for pregnant and nursing mothers for medicinal purposes (Okwu, 2001).

Plants are known to contain antimicrobial components comparable to those of antibiotics (Nascimento et al., 1999). Aqueous solvents such as hot water, cold water, and organic solvents like methanol, hexane, dichloromethane ethanol, acetone, ethyl acetate are widely used to isolate phytochemical antimicrobial compounds (Okoli and Iroegbu, 2005; Pessini et al., 2003). In recent years a number of plants have been screened for the presence of antimicrobial activity (Tomoko Nitta et al., 2001; Meral and Karabay, 2002; Singla and Khan, 2004; Loizzo et al., 2004; Bassam et al., 2004) and for phytochemicals (Kalkar et al., 2005). Phytochemicals have been shown to possess a number of properties like antimicrobial, cure disorders, weight control; induce fever and many more (Eliana, 2006; Sodipo et al., 1991; Konkwara, 1976).
Extraction of bioactive compounds from medicinal plants permits the demonstration of their physiological activity. It also facilitates pharmacology studies leading to synthesis of a more potent drug with reduced toxicity (Pamplona-Roger, 1999; Manna et al., 2000). The active components of herbal remedies have the advantage of being combined with many other substances that appear to be inactive. The active principles of many drugs found in plants are secondary metabolites (Ghani et al., 1990). Plants produce compounds that can be effective antimicrobials if they find their way into the cell of the pathogen especially across the double membrane barrier of Gram negative bacteria (Tegos et al., 2002). Medicinal plants help in alleviating human sufferings. These plants are being integrated to the field of foods as additives, beverages and cosmetics. They are widely used as sweeteners, as bitters, as spices, as natural colouring agents and as insecticides (Purohit et al., 2004).

**PUNICA GRANATUM**

**Nomenclature**

- **Kingdom**: Plantae
- **Division**: Magnoliophyta
- **Class**: Magnoliopsida
- **Subclass**: Rosidae
- **Order**: Myrtales
- **Family**: Lythraceae
- **Genus**: Punica
- **Species**: granatum

**Binomial name**

*Punica granatum*

**Local (vernacular) names**

- In English: Pomegranate; Sanskrit: Dadima; Hindi: Anar; Tamil: Maadhulai; Malayalam: Matalam; Kannada: Daalimbe.
Punica granatum Linn. (Punicaceae) is an erect shrub or small tree native to Asia (Jafri et al., 2000). Punica granatum Linn (Pomegranate) belonging to family punicaceae, which has long been esteemed as food and medicine, and as a diet in convalescence after diarrhoea (Nadkarni, 2000). It is a fruit of great antiquity and is known to have been cultivated in the Middle East more than 5,000 years ago. The plant is found all over India. Pomegranate has been considered important since prehistoric times as an agency of longevity (Ram, 1998). The fruit is a globose berry, crowded by persistent calyx lobes, having a leathery pericarp filled with numerous seeds, which are surrounded by a pink red, transparent, juicy and acidic, pleasantly tasting pulp (Harde et al., 1970).

General Uses

Pomegranate’s use has been mentioned in the ancient literature, including Ayurvedic texts, Ebers papyrus and Greek, Unani and Egyptian documents. It has been used as vermifuge, astringent, bacteriocide, refrigerant, stimulant, stomachic, styptic, hair dye, and to alleviate the adverse effects of asthma, bronchitis, cough, cardiac problems, dysentery, diarrhoea, dyspepsia, fever, inflammation, bleeding disorders, piles, wounds, ulcers, bruises, sores, mouth lesions, stomatitis, vaginitis, respiratory and urinary tract infections, and as a febrifuge to ameliorate malaria and seasonal fevers (Harde et al., 1970; Williamson, 2002; Duke et al., 2002; Nadkarni, 1976). A red dye is obtained from the flowers and also from the rind of unripened fruits (Polunin et al., 1987; Polunin 1945; Grae, 1974). The dye can be red or black and it is also used as an ink (Vines, 1987). A fast yellow dye is obtained from the dried rind (Parmar et al., 1982).

In Ayurvedic medicine pomegranate is considered “a pharmacy unto itself” and is used as an antiparasitic agent (Naqvi et al., 1991), a “blood tonic” (Ladve et al., 1986) and to heal aphthae, diarrhoea, and ulcers (Jurenka, 2008). Pomegranate is a symbol of life, longevity, health, femineity, fecundity, knowledge, morality, immortality and spirituality (Mahdihassan, 1984). Pomegranate peel combined with optimum level of aromatic such as cloves is a most useful remedy in chronic dysentery.
as well as diarrhoea. The rind is an antihelmintic and an astringent and is useful in treating diarrhoea, dysentery and gastralgia (Prashanth et al., 2001; Warrier et al., 2002).

From India (Nagaraju and Rao, 1990), Tunisia (Boukef et al., 1982), and Guatemala (Caceres et al., 1987), reported that dried pomegranate peels are decocted in water and employed both internally and externally for numerous problems demanding astringents and/or germicides, especially for aphthae, diarrhoea and ulcers. Mixtures of pomegranates seed, juice and peel products paradoxically have been reported to not only prevent abortion (Ramirez et al., 1988) but also conception (Gujral et al., 1960; Jochle, 1971; Zhan, 1995). The flowers serve as a remedy for mellitus (Saxena and Vikram, 2004).

Components of Punica granatum

The pomegranate (Punica granatum) tree/fruit can be compartmented: 1.seed, 2.juice, 3.peel, 4.leaf, 5.flower, 6.bark, and 7.roots. The fresh rind of the fruit contains: wax, 0.8; resin, 4.5; mannitol, 1.8; non-crystallized sugars, 2.7; gums, 3.2; inulin,1.0; mucilage, 0.6; tannin, 10.4; gallic acid, 4.0; and calcium oxalate, 4.0%. Pectin occurs to the extent of 2-4 % (Ram, 1998). The root and bark contain tannin (20-22%) and alkaloids (0.5-1%). The seeds contain steroidal oestrogen. The fruit pulp contains protein, carbohydrate, fat, fibre, minerals, oxalic acid and vitamins A, B and C (Lama et al., 2001, Joshi & Joshi, 2001). Edible parts of the pomegranate fruit (80% of total fruit weight) are comprised of 80% juice and 20% seed (Gil et al., 2007). The fresh juice contains 85% water, 10% total sugars, and 1.5% pectin, ascorbic acid, and polyphenolic flavonoids (Aviram et al., 2000). In pomegranate juice, fructose and glucose are present in similar quantities; calcium is 50% of its ash content; and the principal amino acids are glutamic and aspartic acids (Aviram et al., 2000; Cemeroglu et al., 1992; El-Nemer et al., 1990).

Pomegranate fruit is a rich source of two types of poly phenolic compounds: anthocyanins such as delphinidin, cyanidin, and pelargonidin which give the fruit and
Review of Literature

juice its red colour; and hydrolysable tannins such as punicalin, pedunculagin, gallagic and ellagin acid esters of glucose which account for 92% of the antioxidant activity of the whole fruit. The soluble polyphenol content in pomegranate juice varies between 0.2% - 1% (Nar Ben et al., 1996). The seeds contain crude fibers, pectin, sugars and steroid estrogen estron (Moneam et al., 1988), as well as the isoflavone phytoestrogens genistein and daidzein, and the phytoestrogen coumestron (El-Nemer et al., 1990), while the peel extract contains 3 estrogenic compounds-luteolin, quercetin and kaempferol (Elswijk et al., 2004).

The phytochemistry and pharmacological actions of all Punica granatum components suggest a wide range of clinical applications for the treatment of diseases and prevention of cancer, as well as other diseases where chronic inflammation is believed to play an essential etiologic role (Lansky et al., 2007).

Flavonoids and tannins are more abundant in the peels of wild-crafted compared to cultivated fruits (Ozcal et al., 1993). The presence of alkaloids in the peel is equivocal, positive by Dragendorff’s assay, but negative by Mayer assay (Vidal et al., 2003). Leaves contain tannins, glycosides of apigenin, a flavone with progestin (Zand et al., 2000) and anxiolytic (Paladini et al., 1999) properties. The flowers contain flavonoids and tannins (e.g.gallic acid). Pomegranate juice and peel contain substantial amounts of polyphenols such as ellagic tannins, ellagic acid and gallic acid (Loren et al., 2005). It has been used in the preparation of tinctures, cosmetics, therapeutic formulae and food recipes (Finkel et al., 2000) and in this regard pomegranate peel is a good source of antioxidants (Singh et al., 2001).

Extracts of the whole fruit were highly active against Micrococcus pyogens, Staphylococcus aureus, E.coli, and Pseudomonas aeruginosa. They were also very effective against intestinal pathogenic bacilli such as Salmonella paradysenteriae III-Z, S. typhi, S. monetevideo, S. scholtmuelleri and Shigella paradysentriae B.H. Alcoholic extracts of the fruit rind and root bark showed activity against Micrococcus pyogens 60% (Ram, 1998).
Antioxidant Activity

Free radicals have a significant role in the causation of several diseases such as diabetes, cirrhosis, cancer and cardiovascular diseases (Hertog et al., 1993). Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite which results in oxidative stress leading to cellular damage. Thus, compounds or antioxidants that can scavenge free radicals have vital role in the improvement of these diseased conditions (Wilson, 1988).

Free radicals generated in the body can be removed by the body’s own natural antioxidant defences, example: glutathione, catalase etc. Natural antioxidants tend to be safer and they also possess antiviral, anti-inflammatory, anti-cancer, anti-tumour and hepatoprotective properties (Lim and Murtijaya, 2007). Antioxidants play an important role in the alleviation of diabetes due to oxidative stress (Dhanabal, 2005). Oxidative tension is a potent yet non-specific metabolic trigger for both inflammation and angiogenic processes (Hayden and Tyagi, 2004; Karageuzyan, 2005; Kapoor et al., 2005), both of which are key factors in cancer initiation and promotion (Dobrovolskaia and Kozlov, 2005; Garcea et al., 2005; Ohshima et al., 2005).

Pomegranate commonly known as the “jewel of winter” has recently been acclaimed for its health benefits, in particular for its antioxidant potential. Since pomegranate’s antioxidative efficacy clinically may be impaired by poor bioavailability of active compounds (Cerda et al., 2004, 2006), strengths and weaknesses of pomegranate’s antioxidant activity need be considered. In general, comparable juice or extracts from other common fruits show antioxidant activity in vitro inferior to that of the pomegranate (Halvorsen et al., 2002; Kelawala and Ananthanarayanan, 2004; Xu et al., 2005).

Pomegranate is now cultivated mainly in the drier parts of California and Arizona for its fruits exploited commercially as juice products which have been gaining in popularity since 2001, especially because of the appearance of several reports
presenting the antioxidant activities of pomegranate fractions in vitro (Schubert et al., 1999; Gil et al., 2000; Singh et al., 2002). Vitamin C, β-carotene, and α-tocopherol are known to possess antioxidant potential (Prior, 2003; Cai et al., 2004; Kaur et al., 2002). A direct relationship between antioxidant activity and phenolic content of plant extracts has been reported (Ivanova et al., 2005).

**Anti-Inflammatory Activity**

Inflammation, a dynamic process considered as a protective mechanism, leads to a chronic inflammatory state when granulated (Dirosa et al., 1984). During the condition of inflammation associated with pain and fever, arachidonic acid is liberated from the phospholipids fraction of cell membranes and then enzymatically transformed to prostoglandins which sensitize blood vessels to the effect of mediators such as bradykinin, 5-HT and histamines that increase permeability (Al-Rehaily et al., 2001).

Multiplication of small blood vessels as well as proliferation of fibroblasts is the characteristic features at the repair phase of inflammation. The pharmacological properties of the rind extract resemble those of the Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) which are known to possess anti-inflammatory activity. One of the major mechanisms involved in the anti-inflammatory activity of NSAIDs is due to inhibition of prostaglandin (PG) biosynthesis (Vane, 1971).

**Medicinal Properties**

**Anti-atherogenic effect**

Accumulation of cholesterol in macrophages that lead to foam cell formation is the hallmark of atherosclerosis. Major contributors to cholesterol accumulation in arterial cells during atherogenesis include: high plasma cholesterol concentration, increased oxidative stress, reduced serum paraoxonase activity, increased uptake of atherogenic lipoproteins by arterial cells, enhanced macrophage cholesterol esterification rate (Tabas, 1995), and decreased cholesterol efflux from arterial cells (Krieger, 1998).
Pomegranates, which are rich in polyphenolic flavonoids, significantly reduce oxidative stress by inhibiting the formation of oxidised LDL lipoproteins and macrophage lipid peroxidation, and in this mechanism atherogenesis is reduced (Aviram et al., 2000).

**Coronary heart disease**

The effect of pomegranate was studied in patients (n=45) suffering from coronary heart disease and myocardial ischemia, who were randomly assigned into two groups: one group was given pomegranate juice (240 ml/day) for three months and the other group drank a beverage of similar caloric content, amount, flavour, and colour. After 3 months, the extent of stress-induced ischemia decreased in the pomegranate group, but increased in the control group. Thus daily consumption of pomegranate juice may improve stress-induced ischemia in patients who have coronary heart disease (Sumner et al., 2005).

**Hypertension**

Pomegranate juice consumption (50 ml, 1.5 mM of total polyphenols per day, for 2 weeks) by hypertension patients showed a 36% decrement in serum angiogenesis converting enzyme (ACE) activity and a 5% reduction in systolic blood pressure. It follows that pomegranate juice can protect against cardiovascular diseases linked to its inhibitory effect on oxidative stress and on serum and (ACE) angiogenesis converting enzyme activity (Aviram et al., 2001).

**Diabetes and Cholesterol**

The extract of the flowering part of *Punica granatum* may improve post pyramidal hyperglycaemia in patients with type II diabetes and obesity, by inhibiting intestinal alpha-glucosidase activity (Li et al., 2005). The effect of concentrated pomegranate juice consumption (40 g/d for weeks) on lipid profiles of type II diabetic patients (14 women, 8 men) with hyperlipidemia (total cholesterol or triglycerides > or = 200 mg/dl) was evaluated. Pomegranate juice significantly reduced the levels of total cholesterol, LDL-cholesterol, and total cholesterol/HDL-cholesterol. No significant
changes were observed in serum triacylglycerol and HDL-cholesterol concentrations (Esmaillzadeh et al., 2006).

Dental Diseases

A gel containing extracts of *Punica granatum* was effective as adjunctive periodontal therapy in 15 patients with remaining probing pockets of 5-8mm (Sastravaha et al., 2005). The usage of this extracts in three times per day for 15 days was effective for patients afflicted by candidos associated with denture stomatitis (Vasconcelos et al., 2003).

Skin

Kasai et al., performed a double-blind, placenta control trail to clinically evaluate the protective and ameliorative effects of ellagic acid-rich pomegranate extract on pigmentation in the skin after UV-irradiation. Ellagic acid-rich pomegranate extract, ingested orally, has an inhibitory effect on a slight pigmentation in the human skin caused by UV-irradiation. In addition, pomegranate aqueous extracts, especially from pomegranate peels, promoted regeneration of dermis, and lipophilic fractions prepared from pomegranate seed promoted regeneration of epidermis in human skin cells in laboratory conditions (Aslam et al., 2006).

Osteoporosis

According to Mori-Okamoto et al., (2004), it is conceivable that pomegranate juice is clinically effective on a depressive state and bone loss in post menopausal syndrome in women.

Alzheimer’s disease

The neuro-protective properties of pomegranate polyphenols were evaluated in an animal model of Alzheimer’s disease. Transgenic mice with Alzheimer’s like pathology treated with PJ had 50-percent less accumulation of soluble amyloid-beta and less hippocampal amyloid deposition than mice consuming sugar water, suggesting PJ may be neuro-protective. Animals also exhibited improved learning of water maze tasks and swam faster than control animals (Hartman et al., 2006).
Male Infertility

Research in rats demonstrates pomegranate juice consumption improves epididymal sperm concentration, spermatogenic cell density, diameter of seminiferous tubules, and sperm motility, and decreases the number of abnormal sperm compared to control animals. An improvement in antioxidant enzyme activity in both rat plasma and sperm was also noted (Turk et al., 2008).

Antimicrobial

In India, various spices have been traditionally used since ancient times, for the preservation of food products, as they have been regarded having antiseptic and disinfectant properties. The interaction between Punica granatum methanolic extract and 6 antibiotics-chloramphenicol, gentamycin, ciprofloxacin, ampicillin, tetracycline and oxacillin against clinical isolates of multi drug resistant organisms demonstrated that pomegranate extract dramatically enhanced the activity of all antibiotics tested, with synergistic activity detected between pomegranate extract and the antibiotics tested (Braga et al., 2005). A number of in vitro studies have reported the use of plant extracts in combination with antibiotics, with significant reduction in the MICs of the antibiotics against some resistant strains (Darwish et al., 2002).

The curative effect of plant extracts in this combination study has been variably referred to as resistance modifying/ modulating activity (Gibbons, 2004). This speculated that inhibition of drug efflux, and alternative mechanisms of action could be responsible for the synergistic interactions between plant extracts and antibiotics (Lewis and Ausubel, 2006). An extract of Punica granatum rind possessed strong invitro antimicrobial activity against Staphylococcus aureus, E. coli, Pseudomonas aeruginosa and Candida albicans (Navarro et al., 1996). The hot water and other extracts of Punica granatum fruit pericarp showed antibacterial action against organisms such as Salmonella typhi and Vibrio cholerae (Perez et al., 1994; Prashanth et al., 2001).
Antiviral

Tannins from the pericarp of Punica granatum were effective against genital herpes virus (HSV-2). They inhibited replication of the virus, blocked its absorption into cells and had a strong virucidal effect (Zhang et al., 1995). Pomegranate inhibits entry of the HIV virus into human cells. A substance found in pomegranate binds the virus entity which prevents the virus from invading human cells. This makes pomegranate a powerful ingredient for the development of gel-like products for the prevention of sexually transmitted HIV infection (Neurath et al., 2004). Current research seems to indicate the most therapeutically beneficial pomegranate constituents are ellagic acid-ellagitannins (including punicalagins), punicic acid, flavonoids, anthocyanidins, anthocyanins, and estrogenic flavonols and flavones.

Mechanisms of Action

Although pomegranate’s wide-ranging therapeutic benefits may be attributable to several mechanisms, most research has focused on its antioxidant, anti-carcinogenic, and anti-inflammatory properties.

Antioxidant Mechanisms

An in vitro assay using four separate testing methods demonstrated pomegranate juice and seed extracts have 2-3 times the antioxidant capacity of either red wine or green tea (Gil et al., 2000). Pomegranate extracts have been shown to scavenge free radicals and decrease macrophage oxidative stress and lipid peroxidation in animals (Rosenblat et al., 2006) and increase plasma antioxidant capacity in elderly humans (Guo et al., 2008). Research in humans has shown a juice made from pomegranate pulp (PPJ) has superior antioxidant capacity to apple juice.

Anti-carcinogenic Mechanisms

In vitro assays utilizing three prostate cancer cell lines (DU-145, LNCaP, and PC-3) demonstrated various pomegranate extracts (juice, seed oil, peel) potently inhibit prostate cancer cell invasiveness and proliferation, cause cell cycle disruption, induce apoptosis, and inhibit tumour growth. These studies also demonstrated combinations of
pomegranate extracts from different parts of the fruit were more effective than any single extract (Lansky et al., 2005).

**Anti-inflammatory Mechanisms**

Cold pressed pomegranate seed oil (CPPO) has been shown to inhibit both cyclooxygenase and lipoxygenase enzymes *in vitro*. Cyclooxygenase, a key enzyme in the conversion of arachidonic acid to prostaglandins (important inflammatory mediators), was inhibited by 37% by a CPSO extract. Lipoxygenase, which catalyzes the conversion of arachidonic acid to leukotrienes, also key mediators of inflammation, was inhibited by 75% by a CPSO extract. By comparison, an FPJ (fermented pomegranate juice) extract resulted in a 23.8% inhibition of lipoxygenase *in vitro* (Schubert et al., 1999).

The beneficial effect of pomegranate extract reduction of cytokine activity has been shown to occur in patients with periodontitis. Patients experiencing this form of oral inflammation received extragingival chips impregnated with pomegranate peel extract, which resulted in reduced inflammatory cytokines several months post-treatment (Sastravaha et al., 2005).

Most recently, a whole pomegranate methanol extract was also shown to inhibit, in a dose-dependent manner, production and expression of TNFα in microglial cells, in which inflammation had been induced by lipopolysaccharide (Jung et al., 2006). Pomegranate component control of inflammation involves inhibition of both cyclo oxygenases (COX) and lipoxygenases (LOX) enzymes (Schubert et al., 1999) and a decline in prostaglandin release from cells (Polagruto et al., 2003).

**Bacterial Infections**

The only human trials examining the antibacterial properties of pomegranate extracts have focused on oral bacteria (Menezes et al., 2006). However, several *in vitro* assays demonstrate its bactericidal activity against several highly pathogenic and sometimes antibiotic-resistant organisms.
The antimicrobial activity of *Punica granatum* Linn has been widely investigated (Pereira et al., 2006). The findings of several studies, including some relating to inhibition of adherence, suggest that the phytotherapeutic use of this plant might be a viable option in controlling different microbial species. The largest components of the *Punica granatum* L. fruit extract are tannin and polyphenolics (Haslam et al., 1996).

**In Vivo Study**

Animal studies have revealed three possible hypoglycaemic mechanisms for *Punica granatum* extracts. Pomegranate flower extract (PFLE) improved insulin sensitivity and lowered glucose levels in rats as early as 30 minutes post-glucose loading. PFLE also inhibited alpha glucosidase in vitro, thereby decreasing the conversion of sucrose to glucose (Huang et al., 2005). PPE demonstrates significant hypoglycaemic activity in diabetic rats, via enhanced insulin levels and regeneration of pancreatic beta cells (Khalil et al., 2004). Numerous in vitro studies, (Naqvi et al., 1991; Voravuthikunchai et al., 2006; Braga et al., 2005) and two human trials (Menezes et al., 2006) demonstrate the antimicrobial activity of pomegranate extracts.

**Gc-Ms Analysis**

Ellagitannins were purified from fruit husk and analyzed for purity by HPLC and liquid chromatography electro spray ionization mass spectroscopy (LC-ESI/MS) (Seeram et al., 2004). Many researchers have suggested that active constituents found in the herbs are steroids, but no information on pharmaceutical compounds of *Sericocalyx schomburgkii* is available. High performance liquid chromatography (HPLC) and gas chromatography (GC) methods have been used to analyze the steroids from the non-saponifiable portion of liquid extracts from biological samples and foodstuffs (Volin, 2001). Gas chromatography mass spectrometry (Gc-Ms) system has also been used for steroid analysis (Hertmann, 1973; Gaillard et al., 1999).
Modern Uses

Pomegranate derived products now include treatment of acquired immune deficiency syndrome (AIDS) (Lee and Watson, 1998), in addition to use for cosmetic beautification (Kawamada and Shimada, 2002) and enhancement (Curry, 2004), hormone replacement therapy (Lansky, 2000), resolution of allergic symptoms (Watanabe and Hatakoshi, 2002), cardiovascular protection (Shioraishi et al., 2002), ophthalmic ointment (Bruijn et al., 2003), weight loss soap (Guojian, 1995), and as an adjunct therapy to increase bioavailability of radioactive dyes during diagnostic imaging (Amorim et al., 2003).