ZETA POTENTIAL AND POLY DISPERSITY INDEX AS TOOLS FOR STUDYING MEMBRANE DAMAGE –A STUDY ON ERYTHROCYTES AND BACTERIA.

Thesis submitted in partial fulfilment of the requirements for the degree of 'Master of pharmacy'

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This is to certify that the thesis work entitled **"Zeta potential & Poly dispersity index as tools for studying Membrane Damage –A study on erythrocytes & Bacteria"** has been carried out by **Tridib Sarkar** during the session 2014-2016 for the partial fulfilment of the requirements for the degree of **Master of Pharmacy** of **Jadavpur University** under supervision and guidance of myself.

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Tridib sarkar

Examination Roll no:

DEDICATED TO

MY BELOVED PARENTS

&

MY GUIDE

CONTENTS

CHAPTER-1

INTRODUCTION

"Just as chemistry could not have developed without test tubes to hold reacting substances, so organisms could not have evolved without relatively impermeable membranes to surround the cell constituents."

E.N. Harven

DuPraw Cell and Molecular Biology

Nature of drugs and its interaction with biological membrane has been important subject for study since it concerns the drug absorption and/or the direct action of drug to biological membrane. It is difficult to overstate the importance of membranes to living cells; without them life could not exist. The biological membrane regulates the movement of materials into and out of the cell and facilitates electrical signaling between cells.

Other macromolecules also contribute surface charge are in the cell wall and membranes are proteins, phospholipids, teichoic acid, teichuronic acid and Lipopolysaccharides (Moses and Rouxhet, 1990).Cell disruption, which may be performed by physical or chemical processes for recovery of bio molecules such as enzymes and proteins. However, depending on the treatment conditions employed, the wall/cell membrane can be changed (permeabilised) without the release of all of the intracellular components of the cells or without the fragmentation of the cells (Fonseca and Cabral, 2002). When the barrier permeability is altered, the mobility of intra/ extracellular substrates or product is improved. And this improved mobility allows access to a large number of intracellular enzymes and proteins. As in general, most proteins are active inside the cell, and the total amount of an intracellular enzyme can be analyzed after permeabilisation. There are number of procedures for membrane of bacterial membrane permeabilisation have been reported, including the use of surfactants, antibiotics, freeze–thawing, organic solvents, osmotic shock, and polysaccharides (De Leon's et al., 2003). According to report it was found that, non-ionic surfactants are more appropriate for breaking lipid–lipid and lipid–protein interactions than for breaking protein–protein interactions, as is the case of the cationic surfactant (Blaire et al., 2007). Based on a recent survey of literature, it may be mentioned that bacterial membrane is evolving as emerging target for antibacterial drug discovery. Membrane-active agents include the multi targeted lipopeptide, Daptomycin.

Erythrocytes are highly specialized cells transporting oxygen to tissues and removing carbon dioxide. Life span of these cells, devoid of mitochondria and nuclei, as well as

ribosomal mechanism of protein synthesis, is approximately 120 days. The erythrocyte membrane consists of two domains, a lipid bi-layer and the cytoskeleton. The lipid domain is similar structurally to that found in most mammalian cells. The cytoskeleton differs from what is considered cytoskeleton in other cells because it does not contain the structural protein tubulin and is not involved in cell motility or phagocytosis (Tanner. 2002).

The erythrocytes are primarily exposed to xenobiotic which include a diverse range of drugs and chemicals (Mariutti, et al. 2014). According to the available reports, erythrocytes are highly susceptible to endogenous oxidative damage due to presence of both high concentration of polyunsaturated fatty acids (PUFAs) present in the membrane and a high oxygen concentration are known to provide favorable environment for the generation of reactive oxygen species (ROS) (Honzel, et al. 2008). Excess generation of ROS leads to the oxidative stress (owing to an imbalance between the production and native antioxidant defense) and this may often lead to cellular damage. Rapidly increasing environmental pollution is known being considered as a major threat to human health and the pollutants are often found to increase the risk of chronic diseases through enhanced oxidative stress (Liu and Huang 2014).

ROS and free radicals attack erythrocyte membrane and induce oxidation of lipids and proteins (membrane), thereby impairing the rheological properties of blood, which may ultimately alter microcirculation and in turn leads to chronic metabolic disorder (Olchowik, et al. 2012).

The RBC membrane is negatively charged and is surrounded by a fixed layer of cations in the medium (Fig. 1). This fixed layer of cations is surrounded by a cloud-like diffused layer of a mixture of cations and anions. Within the diffused layer, Brownian motion of RBCs and the flow of medium creates a ‘shear’ plane, which separates ions from those ions closely associated with the fixed layer. The potential at the shear plane is defined as the zeta potential (ZP).

Red blood cell (RBC) membranes contain lipids rich in unsaturated fatty acids. RBCs are more frequently exposed to oxygen than other body tissue and, thus, are more susceptible to oxidative damage. Invasion of the RBC membrane by peroxidases may lead to cell hemolysis. Moreover, the hemoglobin in RBCs is a strong catalyst which may initiate lipid peroxidation.

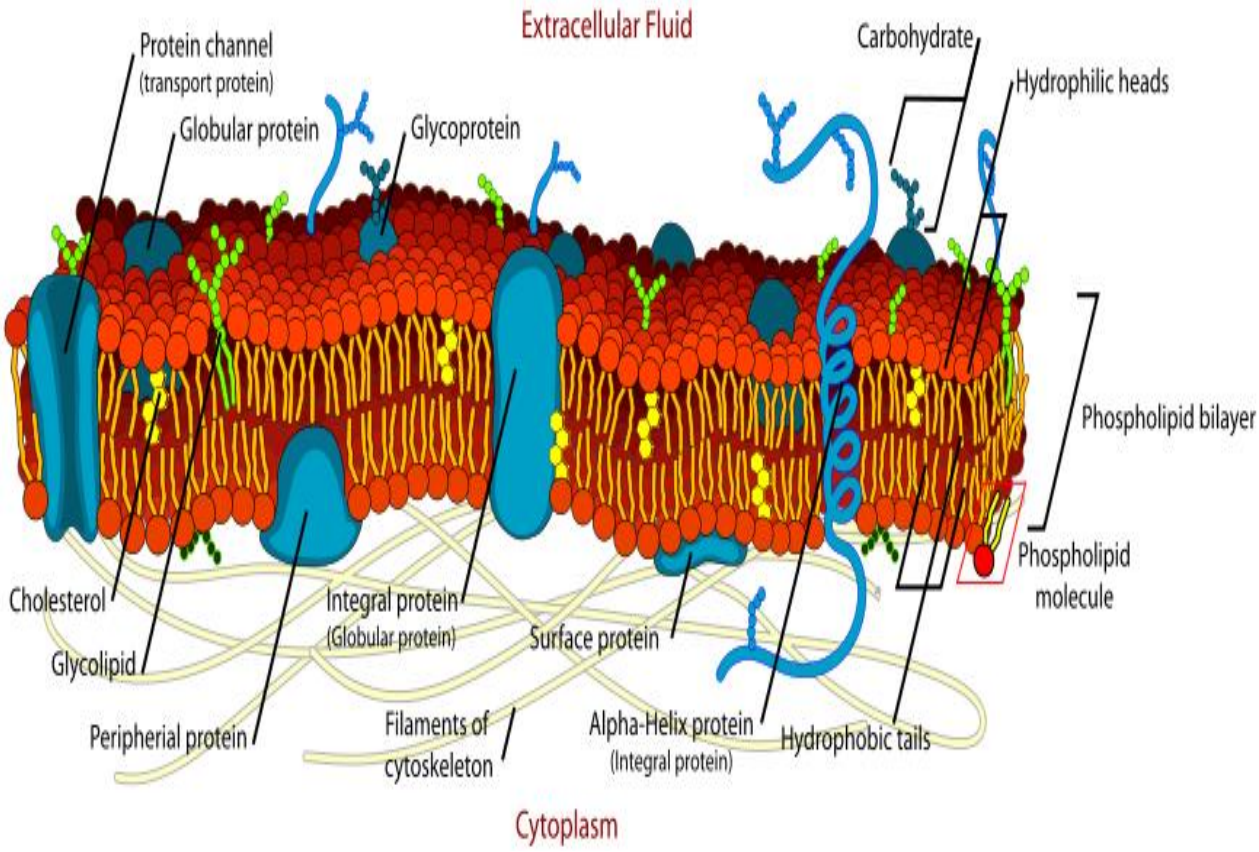

Figure: 1 Membranes comprise a lipid bi-layer plus integral and peripheral proteins (Bolsover et al. 2002).

In healthy aerobic organism, production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is approximately balanced by antioxidant defense systems. The balance is not perfect, however, so that some ROS / RNS mediated damage occurs continuously (Hallowell et al. 1998). The alterations in membrane fluidity and permeability also changes in activities of receptors and membrane-bound enzymes and inhibition of other channels and pumps that is vital to cell metabolism.

In the present investigation an attempts have been made to study the effect of membrane targeting drug on membrane stability, elucidate the possible mechanism. In this present study an attempt was made to evaluate the effect of a cationic detergent and membrane active lipopeptide on both cells and also on erythrocytes and to the perturbation as evident from altered zeta potential with membrane permeability and cell damage. However efforts were also made to elucidate the role of endogenous antioxidants like adhesion on membrane stability.

CHAPTER 2

REVIEW OF LITERATURE

The first cell probably came into being when a membrane formed, enclosing a small volume of aqueous solution and separating it from the rest of the universe. Membranes define the external boundaries of cells and regulate the molecular traffic across that boundary. In eukaryotic cells, they divide the internal space into discrete compartments to segregate processes and components. They organize complex reaction sequences and are central to both biological energy conservation and cell-to-cell communication. The biological activities of membranes flow from their remarkable physical properties. Membranes are flexible, self-sealing, and selectively permeable to polar solutes. Their flexibility permits the shape changes that accompany cell growth and movement (such as amoeboid movement). With their ability to break and reseal, two membranes can fuse, as in exocytosis, or a single membrane-enclosed compartment can undergo fission to yield two sealed compartments, as in endocytosis or cell division, without creating gross leaks through cellular surfaces. Because membranes are selectively permeable, they retain certain compounds and ions within cells and within specific cellular compartments, while excluding others (Boon & Smith. 2002).

Membranes are not merely passive barriers. They include an array of proteins specialized for promoting or catalyzing various cellular processes. At the cell surface, transporters move specific organic solutes and inorganic ions across the membrane; receptors sense extracellular signals and trigger molecular changes in the cell, adhesion molecules hold neighboring cells together. Within the cell, membranes organize cellular processes such as the synthesis of lipids and certain proteins, and the energy transductions in mitochondria and chloroplasts. Because membranes consist of just two layers of molecules, they are very thin—essentially two-dimensional. Intermolecular collisions are far more probable in this two-dimensional space than in three-dimensional space, so the efficiency of enzyme catalyzed processes organized within membranes is vastly increased.

Introduction to bacterial cell

Bacterial cell have a well-developed cell structure, which is responsible for many of their unique biological properties. The cell envelope is composed of the plasma membrane and cell wall. The bacterial cell wall provides structural integrity to the cell and the primary function of the cell wall is to protect the cell from internal turgor pressure caused by the much higher concentrations of proteins and other molecules inside the cell compared to its external environment and in addition, it also confers rigidity and shape of bacterial cells. The bacterial cell wall differs from other organisms by the presence of peptidoglycan which is located immediately outside of the cytoplasmic membrane. Peptidoglycan is made up of a polysaccharide backbone consisting of alternating N-Acetyl muriatic acid (NAM) and N acetyl glucosamine (NAG) residues in equal amounts. Peptidoglycan is responsible for the rigidity of the bacterial cell wall and for the determination of cell shape.

The plasma membrane, also known as the cell surface membrane or plasma lemma, defines the boundary of the cell. The basic function of the cell membrane is to protect the cell fomites surroundings and regulates the movement of materials into and out of the cell and facilitates electrical signaling between cells. It consists of the phospholipids bilayer with embedded proteins. Cell membranes are involved in a variety of cellular processes such as cell adhesion, ion conductivity and cell signaling and serve as the attachment surface for several extracellular structures, including the cell wall, glycocalyx, and intracellular cytoskeleton.

The gram-positive bacteria cell wall is made up of a thick sheath of peptidoglycan and tightly bound with acidic polysaccharides. The cell wall of gram-positive bacteria contains teichoic acid which is a ribitol polymer or glycerol and phosphate embedded in the peptidoglycan sheath. The gram-negative bacterial cell wall is much more complex (Dodge et al. 1962).
In Gram-positive bacteria cytoplasmic membrane (CM) is covered by a thick (20.80 nm) cell wall layer consisting of Peptidoglycan (PG) and adjoining polysaccharides, teichoic acids, teichuronic acids and lipo-teichoic acid. In addition, several Gram-positive bacteria have additional “secondary” cell wall polymers which are involved in the anchoring of bacterial cell surface layers to the bacterial cell surface. Peptidoglycan (PG) is composed of alternating N-acetyl glucosamine and N-acetyl muramic disaccharides, the latter having penta peptide stems. During cell wall biosynthesis these penta peptide stems are cross-linked by creating a cell wall

polymer that is responsible for the maintenance of cell shape and osmotic stability (Daniel & Errington, 2003; Meroueh et al., 2006).

Staphylococcus aureus, is gram-positive cluster-forming coccus (Ryan and Ray, 2004). The staphylococcus genus includes thirty one species and most of them are harmless. Their cell walls are composed of murein, teichoic acids and surface proteins. (Dennis and Steven, 2004).

Bacillus subtilis, is found in soil, water, air, and decomposing plant matter. The cells are spore-forming and are considered to the benign organisms as do not possess traits that cause disease. It is not considered pathogenic or toxigenic to humans, animals, or plants. The potential risk associated with the use of this bacterium in fermentation facilities is low. Bacillus subtilis bacteria secrete enzymes, "such as amylase, protease, pullulanase, chitin's, xylanase, lipase, among others. These enzymes are produced commercially and this enzyme production represents about 60% of the commercially produced industrial enzymes" (Morikawa 2006).

The cell wall of gram negative bacteria consists of a rigid peptidoglycan layer that is much thinner than that of gram-positive bacteria. The cell wall of gram-negative bacteria is also overlaid with an outer membrane, which is a gel-like periplasm in-between the cytoplasmic membrane and the outer membrane. The phospholipids of the cell wall of gram-negative bacteria contain a toxic layer of various proteins, lipoproteins and lipopolysaccharides (LPS) (Madigan et al., 1997). Thus, the major difference between gram-negative and gram-positive bacteria is the thickness of their peptidoglycan layer.

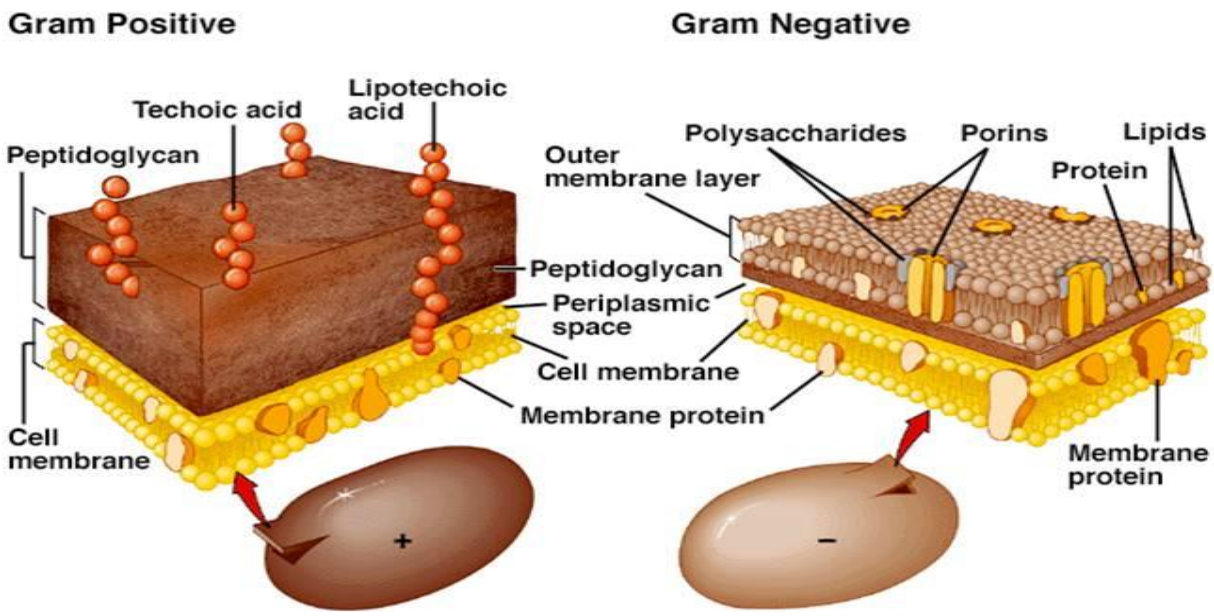

Figure :Gram-positive and gram-negative bacteria

Gram-negative bacteria possess as their outermost layer a special OM that covers the Periplasmic space, the PG layer and the CM. In Gram-negative bacteria the OM and PG are linked to each other with lipoproteins and the OM includes porins, which allow the passage of small hydrophilic molecules. In addition to lipopolysaccharide (LPS), some enterobacteria have entero bacterial common antigens on their OM and in some species capsular polysaccharides are also present. (Alexander and Rietschel, 2001) The outer membrane of Gram-negative bacteria are thick and lipid-rich cell envelope of which act as a permeability barrier and are responsible for the intrinsic resistance of these microorganisms to antimicrobial compounds (Brennan and Nikaido 1995, Nikaido 2001).

The bacterial colony appears golden yellow on agar. S.aureus is a non-motile and non-spore forming facultative anaerobe. It is normally found on human nasal passages, skin and mucous membranes. However, it is categorized as a pathogen to humans as it can cause a wide range of infections, as well as food-poisoning and toxic shock syndrome. (Boris and Filip et al., 2004).

Escherichia coli, which is a well-known gram-negative rod-shaped bacterium. It has flagella for motility, flagella are long appendages which rotate by means of a "motor" located just under the cytoplasmic membrane. The cells may have one, few, or many flagella at different positions on the cell *E.coli* infections among humans can lead to bloody diarrhea, and occasionally to kidney failure, especially in young children and the elderly. The outbreak of *E.coli* infection is frequently associated with eating undercooked beef, mutton and lamb. Studies have that the minimum pH for *E.coli* growth is 4.0 to 4.5(Buchanan and Bagi, 1994) and this is also dependent on the interaction between pH and other growth parameters such as temperature. *E.coli* can be conductively cultured from (21-37)˚ C (Hereunder et al., 1979).

Pseudomonas aeruginosa: The versatile "blue-green pus bacteria" that opportunistically infects people, especially those who are immunocompromised. Pseudomonas rarely causes infection in healthy individuals but it is a major cause of hospital acquired (nosocomial) infections. It tends to infect people with immunodeficiency or burns and those with indwelling catheters or on respirators. Infection with pseudomonas can lead to urinary tract infections, sepsis (blood stream infection) pneumonia, pharyngitis, and many other medical problems. Pseudomonas colonizes the lungs of patients with cystic fibrosis (CF) and contributes to the chronic progressive pulmonary disease and death rate in CF.

Pseudomonas normally resides in the soil, marshes, and coastal marine habitats. It can survive under conditions that few other organisms can tolerate, it produces a slime layer that resists phagocytosis (engulfment), and it is resistant to most antibiotics. Pseudomonas can multiply in an extraordinary assortment of environments including eye drops, soaps, sinks, anesthesia and resuscitation equipment, fuels, humidifiers and even stored distilled water. It has also been reported in kidney dialysis machines. The characteristic color of the pus is due to a bluish pigment (pyocyanin) and a greenish pigment produced by pseudomonas. Published in Nature (Stover et al. 406:959-964, 2000), it was the largest bacterial genome sequenced to that time. The 6.3-Mbp genome contains 5570 predicted genes on one chromosome.

Antimicrobials

An antimicrobial is an agent that kills microorganisms or inhibits their growth. Tietze [F. Enzymatic method] Antimicrobial medicines can be grouped according to the microorganisms they act primarily against. For example, antibiotics are used against bacteria and antifungals are used against fungi. They can also be classified according to their function. Agents that kill microbes are called microbicidal, while those that merely inhibit their growth are called biostatic. The use of antimicrobial medicines to treat infection is known as antimicrobial chemotherapy, while the use of antimicrobial medicines to prevent infection is known as antimicrobial prophylaxis.

Reasons for increasing resistance levels include the following:

• Suboptimal use of antimicrobials for prophylaxis and treatment of infection,
• Noncompliance with infection-control practices,
• Prolonged hospitalization, increased number and duration of intensive care-unit stays,
• Multiple comorbidities in hospitalized patients,
• increased use of invasive devices and catheters,
• Ineffective infection-control practices, transfer of colonized patients from hospital to hospital,
• grouping of colonized patients in long-term-care facilities,
• Antibiotic use in agriculture and household chores, and
• Increasing national and international travel.

Antibiotics

Antibiotics,(literally ‘‘against life’’) also known as antibacterial, are types of medications that destroy or slow down the growth of bacteria or is an agent that either kills or inhibits the growth of a microorganism, according to the US National Library of Medicine says that antibiotics - powerful medicines that fight bacterial infections-can save lives when used properly. Antibiotics either stop bacteria from reproducing or kill them. "Your body's natural defenses can usually take it from there." Antibiotics exert two effects on bacteria: bacteriostatic and bactericidal. Bacteriostatic agents prevent the bacterial cells from growing, as exemplified by the

drug chloramphenicol, and bactericidal agents kill bacteria, as exemplified by penicillin.

Targets of Antibiotics:

Although a large number of antibiotics are used clinically, the variety of targets that they inhibit is limited. Antibiotics are usually classified on the basis of their chemical structure and mode of action.

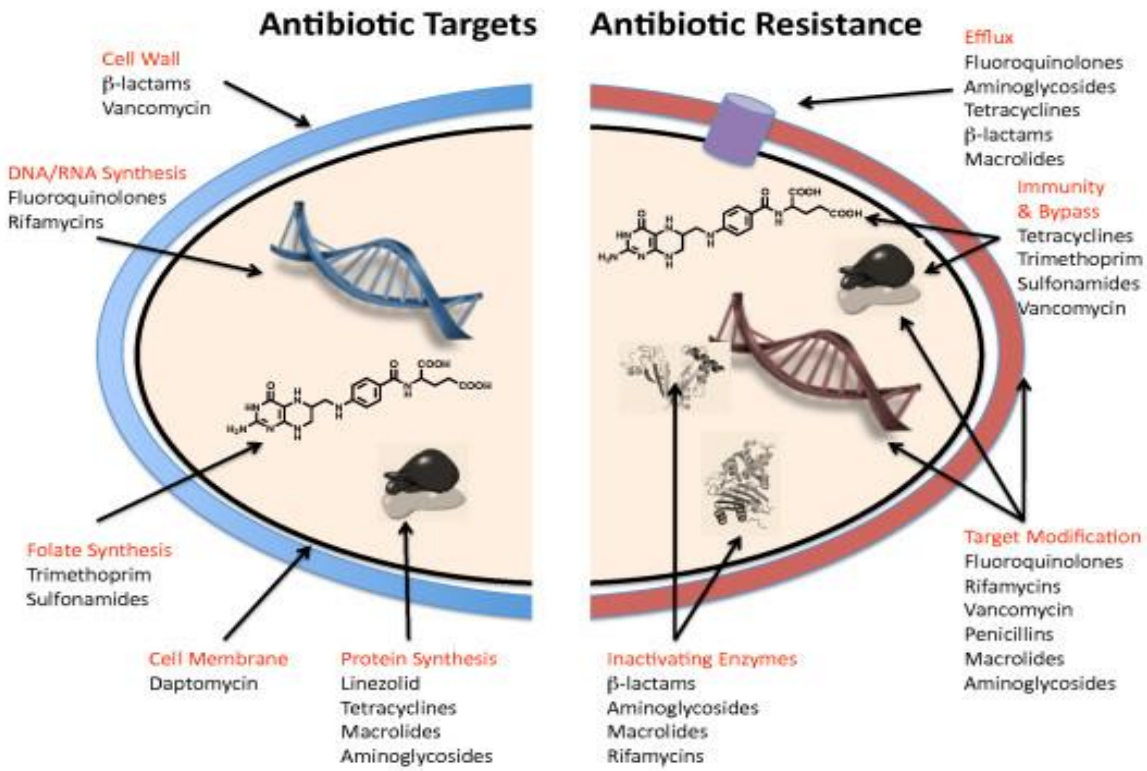

Fig: Anti biotic target and Anti biotic resistance (Davies, J., 1994**).**

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Biomedical importance of membrane

Membranes are asymmetric sheet-like enclosed structures with distinct inner and outer surfaces. These sheet-like structures are non-covalent assemblies that are thermodynamically stable and metabolically active (Figure 2.1). Numerous proteins are located in membranes, where they carry out specific functions of the organelle, the cell, or the organism.

In all cells, the plasma membrane acts as a permeability barrier that prevents the entry of unwanted materials from the extracellular medium and the exit of needed metabolites. Specific membrane transport proteins in the plasma membrane permit the passage of nutrients into the cell and metabolic wastes out of it, others function to maintain the proper ionic composition and pH (~7.2) of the cytosol. The plasma membrane is highly permeable to water but poorly permeable to salts and small molecules such as sugars and amino acids. Owing to osmosis, water moves across such a semi-permeable membrane from a solution of low solute (high water) concentration to one of high solute (low water) concentration until the total solute concentrations and thus the water concentrations on both sides are equal. However, when cells are placed in a hypotonic solution (i.e., one with a lower solute concentration than that of the cell interior), water flows into the cells, causing them to swell. Conversely, in a hypertonic solution (i.e., one with a higher solute concentration than that of the cell interior), water flows out of cells, causing them to shrink. Under normal in vivo conditions, ion channels in the plasma membrane control the movement of ions into and out of cells so that there is no net movement of water and the usual cell volume is maintained (Dowhan. 1997).

Unlike animal cells, bacterial, fungal, and plant cells are surrounded by a rigid cell wall and lack the extracellular matrix found in animal tissues. The plasma membrane is intimately engaged in the assembly of cell walls, which in plants are built primarily of cellulose. In addition to these universal functions, the plasma membrane has other crucial roles in multicellular organisms. Few of the cells in multicellular plants and animals exist as isolated entities; rather, groups of cells with related specializations combine to form tissues. In animal cells, specialized areas of the plasma membrane contain proteins and glycolipids that form specific junctions between cells to strengthen tissues and to allow the exchange of metabolites between cells. Certain plasma-membrane proteins anchor cells to components of the extracellular matrix, the mixture of fibrous proteins and polysaccharides that

provides bedding on which most sheets of epithelial cells or small glands lie. The plasma membranes of many types of eukaryotic cells also contain receptor proteins that bind specific signaling molecules (e.g., hormones, growth factors, neurotransmitters), leading to various cellular responses. Finally, peripheral cytosolic proteins that are recruited to the membrane surface function as enzymes, intracellular signal transducers, and structural proteins for stabilizing the membrane.

	Components (% by weight)				
	Protein	*Phospholipid*	*Sterol*	*Sterol type*	*Other lipids*
Human myelin sheath	30	30	19	Cholesterol	Galactolipids, plasmalogens
Mouse liver	45	27	25	Cholesterol	–
Maize leaf	47	26	7	Sitosterol	Galactolipids
Yeast	52	7	4	Ergosterol	Triacylglycerols, steryl esters
Paramecium (ciliated protist)	56	40	4	Stigmasterol	–
E. coli	75	25	0	–	–

Table: 2.1.A comparative representation components of plasma membrane (Nelson and Cox. 2004)

Factor affecting membrane permeability

Several factors determine the rate of diffusion of a molecule across the membrane depending on the size, polarity and charge of the particular molecule. The rate of diffusion through the bilipid layer for the small, nonpolar molecules is determined by the size and steric configuration or shape of the molecule. The rate of diffusion of uncharged, polar molecules through the bi-lipid layer is modified by molecular size and steric configuration. Lipid solubility, expressed as a partition coefficient, is determined by factors other than simply how easily the molecule dissolves in lipid. For the uncharged, polar molecule to leave the aqueous phase and enter the lipid phase it must first break its hydrogen bonds with water (which requires activation energy in the amount of 5 kcal per broken hydrogen bond) before it can dissolve in the lipid phase.

The number of hydrogen bonds a molecule forms with water is determined by the number of polar groups on the molecule, as well as the strength of the hydrogen bonds formed. For example, the polar hydroxyl (-OH) groups form very strong hydrogen bonds with water, the polar amino ($-NH_2$) groups form weaker hydrogen bonds with water, and the polar carbonyl groups of aldehydes (-CHO) and ketones (-C=O) form even weaker hydrogen bonds with water. Each additional hydrogen bond formed between a polar group and water results in a 40-fold decrease in the partition coefficient, and a resulting decrease in the molecular permeability through the cell membrane. In other words, strongly polar molecules exhibit less lipid solubility due to more polar groups forming hydrogen bonds with the water which hold the polar molecule in the aqueous phase and prevent it from entering the bi-lipid layer of the cell. The reducing ability of the molecule to penetrate the bi-lipid layer and also reduces its diffusion rate across the membrane. Whereas the addition of polar groups decreases penetrating ability and diffusion rate of the molecule, the addition of non-polar groups increases the penetrating ability and diffusion rate of the molecule by allowing the molecules to enter the bilipid layer more easily (Eckert and Randall. 1988).

It may also be mentioned that a rise in the partition coefficient, enhances lipid solubility. Simple diffusion through the bilipid layer exhibits non saturation kinetics, meaning that the rate of influx of penetrating molecules across the membrane increases in direct proportion to the concentration of the solute in the extracellular fluids. Diffusion through aqueous pores does not strictly exhibit non saturation kinetics, for as the extracellular concentration of the molecules increases, the aqueous channels can become filled with solute inhibiting free diffusion across the bilipid layer. Therefore, at low extracellular solute concentrations, the rate of influx of solutes through the pores increases in direct proportion to the concentration of the solute in extracellular fluids, but at high extracellular solute concentrations the influx of solutes through the pores decreases slightly. The carrier mediated route exhibits saturation kinetics, wherein the rate of influx reaches a plateau beyond which a further increase in solute concentration does not increase in the rate of influx. This is because the number of carriers, the rate at which carriers can react with molecules, and the actual transport of the molecule across the membrane is limited. Therefore, the rate of carrier-mediated transport increases in direct proportion at lower extracellular solute concentrations, then reaches a maximal level when the carrier molecules are saturated (Giese A C. 1963).

Compounds that disrupt the cross-bridging between lipids a molecules increase the permeability of the OM. The cross-bridging can be disrupted in at least two ways. At relatively high concentrations, metal chelators, such as EDTA, compete with lipid A for divalent cations and increase OM permeability (Leive L. 1974). At much lower concentrations, compounds that selectively associate with lipid A also increase permeability.

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Mechanism for diffusion across membrane:

The rate at which a molecule diffuses across a membrane depends on its size and its degree of hydrophobicity. Hydrophobic substances such as gases and steroid hormones diffuse across membranes easily. Due to the fact that they are repelled by the hydrophobic interior of the bilayer, polar molecules do not diffuse across the bilayer as easily, unless they are very small and uncharged (e.g. water and EtOH) (Horton et al. 2006).Lipid bilayers are much less permeable to larger polar molecules, and are virtually impermeable to ions, which are surrounded by a cage of water.

In order to cross the hydrophobic interior the bilayer, water soluble molecules (those that are either charged or have polar groups) and large molecules require the action of membrane transport proteins. These integral membrane proteins provide a continuous protein-lined pathway through the bilayer. There are two classes of membrane transport proteins which are as follows: carrier proteins, which literally carry specific molecules across, and channel proteins, which form a narrow pore through which ions can pass. Channel proteins carry out passive transport, in which ions travel spontaneously down their gradients. Some carrier proteins mediate passive transport (also called facilitated diffusion), while others can be coupled to a source of energy to carry out active transport, in which a molecule is transported against its concentration gradient.

Membrane transport proteins are important pharmacologically for two reasons. First, some drugs exploit endogenous membrane transport processes to enter or exit cells. Second, membrane transport proteins are major drug targets (Horton et al. 2006).

Large macromolecules (e.g. proteins, viruses, lipoprotein particles) require more complex mechanisms to traverse membranes, and are transported into and out of cells selectively via endocytosis and exocytosis (secretion). Interestingly, endocytosis and exocytosis are not only important for the import/export of large molecules. Often, essential small molecules that are hydrophobic or toxic (e.g., iron) travel through the bloodstream bound to proteins, which enter and exit cells via these mechanisms (Horton et al. 2006).

Surfactants, antibiotics and organic solvents are the most commonly used substances for membrane cellular permeabilisation, and surfactants are attractive because of the safety issues associated with the use of solvents. Here concentration of the surfactant and antibiotic are crucial factors for cell permeabilisation. However, their influence strongly depends on the composition of the cell wall/membrane as well as the location of enzyme to be assayed (Galabova et al. 1996). According to (Blaire et al., 2007), surfactants solubilize membrane proteins by mimicking the lipid-bilayer environment and cationic surfactants such as CTAB (cetyl trimethyl ammonium bromide) can promote electrostatic and hydrophobic interactions with the proteins, whereas non-ionic surfactants such as Triton X-100 only produce hydrophobic interactions (Rocha, 1999).

Peptidic antibiotics such as polymixin B, colistin and salinomycin. Antibiotics like the family of β-lactam, penicillin's and cephalosporin's, act primarily at the level of the bacterial membrane by inhibiting membrane proteins associated with cell wall synthesis and cause cell membrane permeabilisation by leading to an increase in membrane porosity and depolarization, and cellular disruption by inhibiting membrane proteins related with cell wall synthesis(Nikaido and Norm ark, 1987).As in metabolically active bacteria with intact cytoplasmic membranes, a trans-membrane potential develops due to the action of integral proteins, selectively group and transport nutrients, H+ ions, physiologically relevant substrates and inorganic ions across the lipid bilayer. Due to molecular disruption of the membrane, the enzymes causing polarization via transport, or by disrupting protein-protein interactions within the membrane (Chia et al., 1993). In general, the polarization and membrane potential of the cell membrane disappears with molecular disruption and perturbation, where pores develop and ions cross freely, cancelling molecular charges and slowing or stopping physiological mechanisms, such as DNA and

protein synthesis. Similarly poly functional antibiotics, also alter cell membrane permeability and membrane potential. Among the above given antibiotic the Polymixin antibiotics that target cell membranes are being used more frequently to treat multidrug-resistant Gram-negative infections.

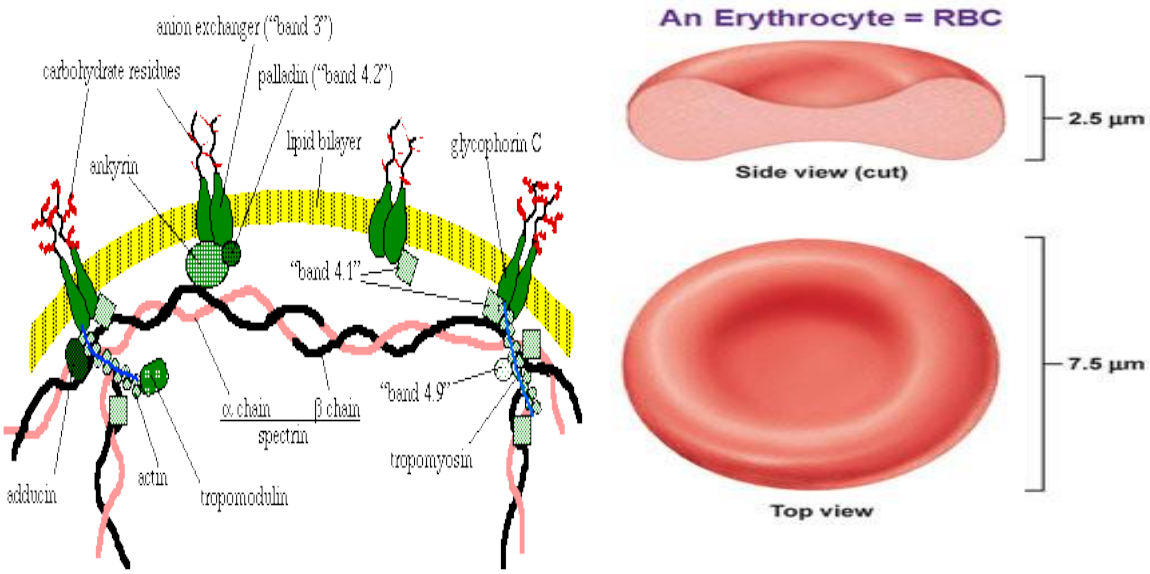


Figure : Erythrocyte [Davis K J and Goldberg A L.]

Assessment of cell membrane damage:

The outer membrane serves as a physical and functional barrier for gram negative bacteria. In recent years considerable efforts have been made to understand the functions, synthesis, composition, and structure of outer membrane components (Dirienzo et al., 1973).The gram-negative cell envelope consists of two membranes separated by a layer of ptidoglycan and a cellular compartment called the periplasm. The innermost, cytoplasmic membrane is generally a phospholipids bilayer liberally studded with a wide variety of polypeptides. The major functions of cytoplasmic

membrane proteins are in energy generation, in active and facilitated transport of nutrients and export of toxic byproducts, and in enzymatic synthesis and translocation of cell envelope components (Dirienzo et al. 1973). The cytoplasmic membrane serves as a major barrier for hydrophilic or charged molecules but it is generally accepted that even moderately hydrophobic molecules can enter into or even cross the lipid bilayer at growth temperatures (Nikaido, 1976). The periplasm is probably a matrix of polypeptides and saccharides with net negative charges. The outer membrane is an unusual biological membrane in that its outer monolayer contains lipopolysaccharide (LPS) as its major lipidic molecule while the inner leaflet contains phospholipids rather than LPS. LPS is an amphiphilic molecule containing a hydrophobic region (Lipid A. Also known as endotoxin) that has 5 or 6 fatty acids linked to di glucosamine phosphate. LPS carries a net negative charge resulting in the strong negative surface charge of gram-negative cells. One of the most important features of LPS is that it appears to be anchored in the outer membrane by binding to outer membrane proteins, possibly through hydrophobic interactions with Lipid A, and by noncovalent cross-bridging of adjacent LPS molecules with divalent cations. Compounds that disrupt the cross-bridging between lipids a molecules increase the permeability of the OM. (Robert et al. 2006)

Drug resistance : Drug resistance is The ability of bacteria and other microorganisms to withstand a drug that once stalled them or killed them. More commonly, the term is used in the context of resistance that pathogens have "acquired", that is, resistance has evolved.

"Drug resistance develops naturally, but careless practices in drug supply and use are hastening it unnecessarily." **- Center for Global Development**

"The overuse of antibacterial cleaning products in the home may be producing strains of multi-antibiotic-resistant bacteria." - **Better Health Channel - Australian Government**

Bacteria are capable of not only altering the enzyme targeted by antibiotics, but also by the use of enzymes to modify the antibiotic itself and thus neutralize it. Examples of target-altering pathogens are Staphylococcus aureus. while examples of antibiotic-modifying microbes are Pseudomonas aeruginosa .

Antimicrobial resistance

Antimicrobial resistance is resistance of a microorganism to an antimicrobial drug that was originally effective for treatment of infections caused by it and has ability of microbes, such as bacteria, viruses, parasites, or fungi, to grow in the presence of a chemical (drug) that would normally kill it or limit its growth.

The evolution of resistant strains is a natural phenomenon that occurs when microorganisms replicate themselves erroneously or when resistant traits are exchanged between them. The use and misuse of antimicrobial drugs accelerates the emergence of drug-resistant strains. Poor infection control practices, inadequate sanitary conditions and inappropriate food-handling encourage the further spread of antimicrobial resistance.

Membrane active drugs and their role in distributing bacterial cell :

Daptomycin

Daptomycin is a novel cyclic lipopeptide antibiotic that provides rapid bactericidal activity against gram-positive pathogens in vitro, including methicillin-susceptible Staphylococcus aureus, methicillin-resistant S. aureus, vancomycin-resistant S. aureus, penicillin-resistant Streptococcus pneumoniae, and ampicillin- and vancomycin-resistant enterococci.

Daptomycin is a cyclic lipopeptide derived from Streptomyces roseosporusas a fermentation product [Galabova, D., 1996] . It consists of a highly lipophilic decanoic acid side chain linked to the N-terminal tryptophan of a cyclic 13-member peptide (figure 1) [2, 3]. The precise mechanism of action is not completely understood; it is known to act at the cytoplasmic membrane, binding to the membrane via a calcium-dependent insertion of its lipid tail [Filipe V, Hawe A, Jiskoot]. It is only active against gram-positive bacteria because it is unable to penetrate the outer membrane of gram-negative bacteria [Powers, J.P., Hancock, R. E., 2003]Current understanding of its mechanism of action suggests that it forms an ion-conduction structure, resulting in an efflux of potassium (and possibly other ions) with an associated dissipation of the ion concentration gradient [Kohen R and Nyska A. Toxicol. Pathol. 2002]. Large molecules are not released from the cytoplasm. Cell death occurs as a result of widespread dysfunction of macromolecular synthesis, including synthesis of DNA,

RNA, and proteins; cell lysis does not occur . Notably, the activity of daptomycin is dependent on the presence of calcium ions, which directly impacts the conditions required for in vitro susceptibility testing [Cheung L M, Cheung K P C.].

POLYMYXINS B:

Polymixins (Figure1.4) are antibiotics, with a general structure consisting of a cyclic peptide with a long hydrophobic tail, After binding to lipopolysaccharide (LPS) in the outer membrane of gram-negative bacteria, polymixin disrupt both the outer and inner membranes and affecting cellular permeability in both growing and resting cells. The hydrophobic tail is important in causing membrane damage, suggesting a detergent-like mode of action. Due to differences in the amino acid derived cyclic portion in structure of the molecule designate the different form of Polymixins. Polymixin B1 and E1 are among the most studied and used clinically. According to Clausell, (2003) it was found that at low concentrations, polymyxin B1 binds to the outer membrane of E.coli, slightly disturbing it, while higher concentrations cause further incorporation into the membrane and finally causes membrane permeable which further leads to cell death.

CTAB: CTAB is a cationic detergent, soluble in H2O and readily soluble in alcohol. CTAB is commonly used in the preparation and purification of genomic DNA from bacteria including DNA mini preps for sequencing. CTAB complexes with both polysaccharide and residual protein.

Cetyltrimethylammonium bromide is a cationic surfactant. It is widely used as topic antiseptics, and may be found in many household products such as shampoos, hair conditioning product and cosmetics. According to the Directive 67/548/EEC, this chemical is not classified. Based on the biodegradability screening tests available (MITI and Gracia et al), CTB is considered to be readily biodegradable at low concentration (2.5mg/l). However at high concentration (10mg/l) a very slow and incomplete degradation were observed.

CHAPTER -3

MATERIALS AND METHODS

Materials Used

- Chemicals utilized in the present investigational work were
- Cetyl trimethyl Bromide (CTAB) (Merck),
- Polymixin B (Hi-media),
- Daptomycin (agiala –a strides enterprise)
- (NPN) 1-N-phenylnaphthylamine (HIMEDIA),
- For phosphate buffer solution (PBS) : reagent used is K2HPO4 (Potassium Phosphate Dibasic Anhydrous) (SRL) and KH2PO4(Potassium dihydrogen Phosphate GR(MERCK),
- Muller Hinton broth (HI Media)
- Sterile 96-well microtiter plates (Future Bio-science.)
- Dimethyl sulphoxide (DMSO)(Merck),
- Ethylene Diamine Tetra Acetic acid (EDTA) –(SRL),
- Glutathione (GSH) (SRL)
- Muller Hinton broth (HI Media) (for checking % of lysis).
- Analytical grade Magnesium chloride (Merck).
- For the whole procedure the water used was deionized or Milli-Q.
- 2 ml violet cap colored vaccutech that contains k3 EDTA (anti-coagulant).

Bacterial strains

The test microorganisms utilized in this study included both Gram-positive (***Staphylococcus aureus*** MTCC 96 and ***B.subtilis*** MTCC 441) and Gram-negative bacteria (***Escherichia coli*** MTCC 2939 and ***P.aeruginosa*** ATCC 278531).

The strains were maintained on nutrient agar (NA) plate and were stored at 4 °C. A single isolated colony was picked from this plate and transferred to Mueller–Hinton Broth (MHB) and was incubated at 37 °C. Density of the broth was adjusted to 0.5 McFarland standard with MHB (Andrews 2001).

Preparation of stock solution

Stock solution of 1mg/ml of CTAB, Polymyxin B and Daptomycin were prepared individually in 0.5 mM potassium phosphate buffer solution (pH7.4). Different concentration of CTAB (0.3, 0.6, 30, 60, 120 µg/ml), Polymyxin B (0.3, 0.6, 30, 60, 120 µg/ml) Daptomycin (0.3, 0.6, 30, 60, 120 µg/ml) were utilized to carry out the present investigational work.

Preparation of bacterial suspension and treatment

100 µl of bacterial culture was freshly inoculated in 5 ml of Mueller Hinton Broth (MHB; Hi-media) and incubated at 37 °C for 360 min, where final bacterial concentration of ~1.1 × 10^9 colony forming units/ml (CFU/ml) was reached (mid log phase; 0.4 at OD_{590}), indicating satisfactory growth of bacteria (Soon et al 2011). The bacterial suspensions were centrifuged at 10,000 rpm, (20 min), the supernatant was discarded and the cell pellets were washed five times with 0.5 mM potassium phosphate buffer saline (pH 7.4)/0.9% normal saline. The bacterial cell suspension was prepared by re-suspending the cell pellet in 0.5 mM potassium phosphate buffer solution (pH 7.4). The OD_{590} of the final dispersion varied between 0.12 and 0.15 (Kłodzińska et al. 2010). The washed bacterial cell suspensions were incubated with different concentration of CTAB (0.3, 0.6, 30, 60, 120 µg/ml), polymyxin B (0.3, 0.6, 30, 60, 120 µg/ml) and Daptomycin (0.3, 0.6, 30, 60, 120 µg/ml) for different time periods (30, 60, 90 and 120 min).

Preparation of 0.9% (w/v) normal saline:

0.9 gm of NaCl is dissolved in 100 ml of Mili Q water. Volume that we used to make was near about (50-75) ml.

Preparation of RBC cell suspension and treatment

First (goat) blood is collected from the slaughter house on a 2ml vaccutech anti-coagulant blood carrier. Then centrifuge blood on a 1.5 ml eppendorf at 2000 rpm for 10 min , discard the supernatant and then wash the pellets of RBC using phosphate buffer saline for 2 times at 2000 rpm for 10 min. at last discard the supernatant finally and get the final conc. Of RBC cell suspension by mixing 30µl of RBC pellets along with 30ml of phosphate buffer saline in a 50 ml falcon tube. After

that, in a 6 well plate, 5 ml RBC cell suspension on each well are distributed and different conc. Of drug (0.3, 0.6, 5, 10, 30 mcg /ml) are put on cell suspension and then for time dependent incubation the plate was placed on a shaker incubator. During this period on different time interval (30, 60, 90, and 120) min reading of zeta potential, PDI result on nano zetasizer.

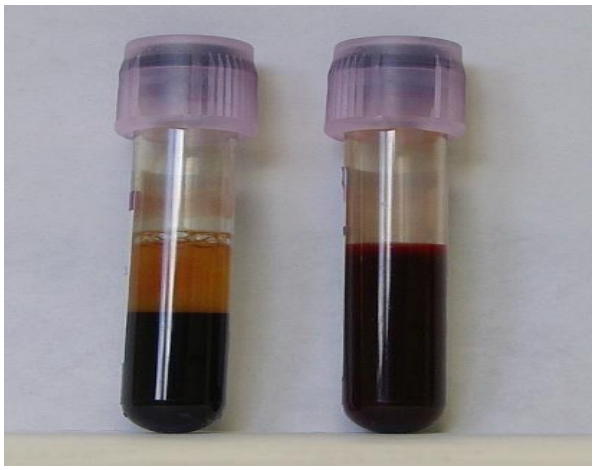


Figure: RBC cell suspension (https://commons.wikimedia.org/wiki/File:Hemolysis.jpg)

Estimation of % of lysis of RBC:

At first (goat) blood is collected from the slaughter house on a 2ml vaccutech anti-coagulant blood carrier. Then centrifuge blood on a 1.5 ml Eppendorf at 2000 rpm for 10 min , discard the supernatant and then wash the pellets of RBC using phosphate buffer saline for 2 times at 2000 rpm for 10 min. at last discard the supernatant finally and get the final conc. Of RBC cell suspension by mixing 30µl of RBC pellets along with 30ml of phosphate buffer saline in a 50 ml falcon tube. After that, in a 6 well plate, 5 ml RBC cell suspension on each well are distributed and different conc. Of drug (0.3, 0.6, 5, 10, 30 mcg /ml) are put on cell suspension and then for time dependent incubation the plate was placed on a shaker incubator. During this period on different time interval (30, 60, 90, and 120) min reading of RBC cell % lysis taken, for that few Eppendorf was collected and marked according to different conc. of drug that was dissolved on 6 well plate for incubation. Now for double distilled water and normal saline was taken on different Eppendorf (100 µl) each and 200 µl of cell suspension (time dependent incubated) put on those eppendorf. Then those eppendorf s were put on for centrifugation at 2000 rpm for 5

mins. Then from those eppendorf s 200 µl of sample (supernatant) was pipette out and put on 96 well quartz plate and at 540 nm on The Multiskan GO UV/Vis microplate spectrophotometer reading of (NS/DW) is taken.

Multiskan GO microplate spectrophotometer is a handy tool for virtually all Photometric research applications. Multiskan GO as stand-alone with straightforward internal software for quick and simple measurements. Multiskan GO microplate spectrophotometer supports endpoint, kinetic and spectral scanning assays. For analysis purpose here we have used 96- Microplates with and without lids, and all major cuvette types. The reading speed of Multiskan GO is exceptionally fast; all wells in a 96-well plate are measured in less than 6 seconds. A broad wavelength range with the UV area, path length correction as well as fast spectral scanning makes it an ideal tool for any photometric research application, including DNA, RNA and protein analysis.

Visual and comprehensive assay setup with SkanIt Software

The logical and truly user-friendly interface of the SkanIt Software makes the instrument control and assay setup easy even for advance d applications. The SkanIt Software offers a comprehensive selection of inbuilt calculations, including quantitative curve fit, qualitative classification, kinetic calculations, spectral reduction and parallel line analysis*) (PLA) to make data reduction very straightforward. In addition, any measurement or calculated data in the SkanIt Software can be automatically exported to Excel with just one mouse click. The same language selection as for the internal software is available also for the SkanIt Software. It simply speaks the language.

Fig: Multiscan Go Spectrophotometer (http://www.news-medical.net)

Estimation of Zeta potential

The Zeta potential depends on the composition of the cell surface as well as on the nature of the surrounding medium. Several factors such as conductivity (salt concentration) and pH of the medium govern the adsorption of ions onto bacterial and RBC cells and influence the degree of ionization of charged moieties on the cell surface (Soon et al. 2011). Keeping these in view, the Zeta potential measurements were performed with 0.5 mM potassium phosphate buffer solution (pH 7.4) to minimize any influence of pH. The Zeta potential was measured with the help of a Zetasizer Nano ZS 90 device (Malvern, UK), equipped with Helium–Neon laser (633 nm) as a source of light, with the detection at 90 degree scattering angle at room temperature (28 °C). Each of the experiments was carried out under identical experimental condition (n = 5). Zeta potential was also recorded for autoclaved (at 121 °C, at 15 psi, for 20 min) (Martinez et al. 2008) and GSH treated bacterial and RBC cells.

FIGURE: Nano zetasizer (http://www.news-medical.net/Zetasizer-Nano-ZSP-Electrophoresis-Systems-from-Malvern)

Nano sight

Nano Sight NS300 uses the technology of Nanoparticle Tracking Analysis (NTA). Nanoparticle tracking analysis (NTA) is a system for sizing particles from about 30 to 1000 nm and ability to determine the concentration of particles in the solution within the concentration range from 10E+7 to 10E+9 particles/ ml depending on sample type. This unique technology utilizes the properties of both light scattering and Brownian motion in order to obtain the size distribution and concentration measurement of particles in liquid suspension. A laser beam is passed through the sample chamber, and the particles in suspension in the path of this beam scatter light in such a manner that they can easily be visualized via 20x magnification microscope onto which is mounted a camera. A highly sensitive digital camera records a video of this motion, after which software tracks each visualized/detected particle and calculates its diameter. The camera operates at 30 frames per second (fps), capturing a video file of the particles moving under Brownian motion. The software tracks many particles individually and using the Stokes-Einstein equation calculates their hydrodynamic diameters.

Figure: Nanosight NS300 (http://www.atascientific.com.au)

Estimation of Particle movement and size distribution:

For the estimation of particle movement RBC cell suspension was prepared as mentioned before, and put CTAB (5 µg/ml) for treatment along with a comparison with blank RBC cell suspension. At first 1 ml of blank RBC cell suspension was seen and then the same cell suspension treated with CTAB 20 µl from the stock. The CTAB treated particles viscosity was changed along with merged particles .The 3D band was also shifted and the CTAB effect was clearly visible with its lysis power. The visualization of particles was seen on 600 nm of fluorescence (for blank). Here the threshold was changed and the small particles were omitted to solve the mean problem .On a 3D view different bands are observable (through light scattering property). Here we saw CTAB treated RBC cell (small) particles were moving fast (on 195 nm of fluorescence) .Mixed Particles were distinguishable .High resolution of particles were detected. Size distribution was seen particle by particle.

Estimation of polydispersity index

The PDI of the samples was measured in a cuvette at 90 degree scattering angle, with the Zetasizer for both untreated and (CTAB, Polymyxin B and Daptomycin) treated bacterial cells along with RBC cell suspension this experiment was conducted at room temperature (28 °C).

Measurement of permeability with 1-N-phenylnaphtylamine (NPN)

The outer membrane permeability of P. aeruginosa E. coli and S. aureus and B. subtilis was determined according to Hollander (Helander and Mattila-Sandholm 2000). A 10 mM stock solution of NPN (in ethanol) was diluted to a concentration of 20 µM with 0.5 mM potassium phosphate buffer (pH 7.4) solution. The fluorescence of the samples was measured at an excitation and emission wavelength of 350 and 420 nm, respectively (Spectra Max M5). NPN permeability assay was also carried out for autoclaved and CTAB, Polymyxin B, Daptomycin treated bacterial cells.

Effect of Anti-microbial derivatives on Erythrocyte

Direct Hemolytic activity:

Direct hemolytic activity on washed erythrocytes (goat blood) is determined according to a modified method of Boman and Kaletta (1957). Blood is drawn freshly by puncturing the retro orbital plexus of ether anesthetized rat in a citrated tube (1 ml of 3.8% sodium citrate dehydrate per 5 ml of blood). The erythrocyte is collected following centrifugation of the citrated (2000 rpm; 4°C; 10 min). The erythrocyte then washed thrice with normal saline. Finally 0.1% (v/v) RBC suspension in normal saline is prepared (25 mcl RBC pellet in 25 ml of PBS) for the assay. Erythrocyte suspensions (final concentration of 0.1%; 25 ml) were incubated with specified concentrations of samples (in terms of protein equivalent) at 37ºC for (30, 60, 90,120) mins in a shaking incubator (100 cycles/minute).By this time of incubation different conc. Of drug treated RBC cell suspension was again incubated, after centrifugation on 96 well quartz plate the absorbance of supernatant (A) at 540 nm was read. Similarly another aliquot of the reaction mixture was diluted with distilled water to yield complete hemolysis and the absorbance of the supernatant (B) after centrifugation was measured at 540nm. The percentage hemolysis exhibited by each sample was calculated by equation (A/B) x 100.

Determination of reduced glutathione (GSH) content

Glutathione was estimated using the method of Tietze (1969) with some modifications. 100 µl of 25% Trichloroacetic acid (TCA) was added to 0.4 ml of lysate. The solution was then centrifuged at 5000 rpm at 4°C for 15 min. 100 µl of protein free solution was then taken from the supernatant to which 200 µl of 0.6 mM dithionitrobenzidine (DTNB) solution in 0.2M phosphate buffer, pH 8.0 was added. Blank containing 100 µl
of 5% TCA and glutathione standard in the range of nomograms were run simultaneously. The optical density (O.D.) was taken at 412 nm. The amount of glutathione was then calculated from the standard curve, and the results expressed as Nano moles of glutathione per mg protein. Protein estimation was done using the Bradford's method (Bradford. 1976). Each experiment was done in duplicate.

Effect of GSH on the lytic property of the CTAB

Erythrocyte suspension (0.1 %) is prepared in the same manner as described in the direct hemolytic assay. Antioxidant like, GSH are incubated for 30 minute at different concentration (0.3, 0.6,5,10 & 30 µg/ml). After 30 minute 10µg/ml of compound was incubated at 37 °C for 4 h in shaking incubator, an aliquot of the reaction was diluted 10 times with PBS and centrifuged at 2000 rpm for 10 min, the absorbance of its supernatant was then read at 540 nm by a spectrophotometer (Absorbance A). Similarly, another aliquot of the reaction mixture was diluted with distilled water to achieve complete hemolysis and the absorbance of the supernatant obtained after centrifugation was measured at 540 nm (Absorbance B). The percentage hemolysis exhibited by each sample was calculated by equation (A/B) x 100.

Microscopic Evaluation:

Microscopic evaluation of RBC cell suspension is done using digital microscopy. It is a fully integrated, digital, inverted imaging system for four-color fluorescence and transmitted-light applications. It is powerful, yet easy-to-use and delivers high-definition images with exceptional convenience. The unique light cubes, sensitive camera, and precision-engineered optical system make this microscopic system ideal for both demanding and routine fluorescence imaging applications of the RBC and each cell is properly describable on slides containing RBC suspension.

Figure: **EVOS® FL Imaging System** (https://www.thermofisher.com)

Statistical analysis

All analyses were performed in triplicate. The data were recorded as means standard deviations. One-way analysis of variance (ANOVA) and Dennett multiple comparisons were carried out to test for any significant differences between the means. Differences between means at 5% ($P<0.05$) level were considered significant.

CHAPTER-4

RESULTS

I. Effect of CTAB, Polymyxin B and Daptomycin on gram positive and gram negative bacteria:

In this study we have determined the MIC of the compounds against the different bacterial strains and then based on the MIC the effect of these compounds were evaluated on the different strains for ascertaining their effects on Zeta potential (ZP), Polydispersity Index (PDI) and NPN uptake.

1. Effect of CTAB on *S. aureus*, *B. Subtilis*, *E.coli* and *P. aeruginosa*

1.1. Minimum inihibitory concentration:

Table 1a. Minimum inihibitory concentration (MIC) of CTAB against Gram positive and Gram negative organisms

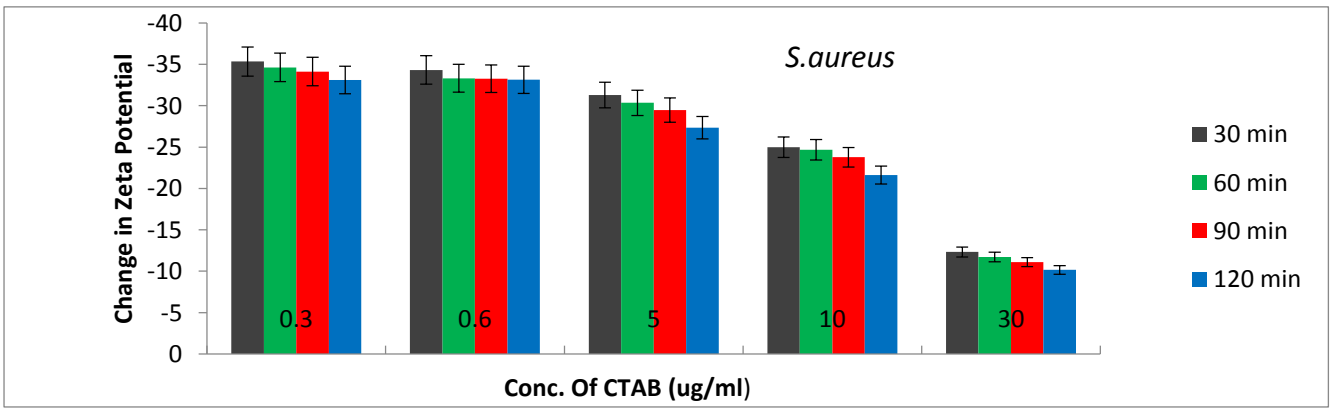
Agents	Organisms studied	Concentration(µg/ml)
CTAB	*S.aureus*	0.625
	B.subtilis	0.625
	E.coli	0.312
	P.aeruginosa	0.312

1.2. Zeta Potential (ZP) measurement

Table 1b: Effect of CTAB on the Zeta Potential (ZP) of the *S.aureus* (gram positive)

Conc. Of CTAB (µg/ml) ZP of S .aureus (-35.6)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	-35.34±1.7	-34.63±1.7	-34.13±1.7	-33.1±1.6
0.6	-34.32±1.7	-33.32±1.6	-33.275±1.6	-33.13±1.6
5	-31.29±1.56	-30.35±1.4	-29.47±1.4	-27.35±1.3
10	-24.986±1.24	-24.68±1.2	-23.77±1.2	-21.63±1.08
30	-12.32±0.61	-11.72±0.5	-11.1±0.5	-10.155±0.5

INFERENCE- CTAB causes changes in Zp of S.aureus

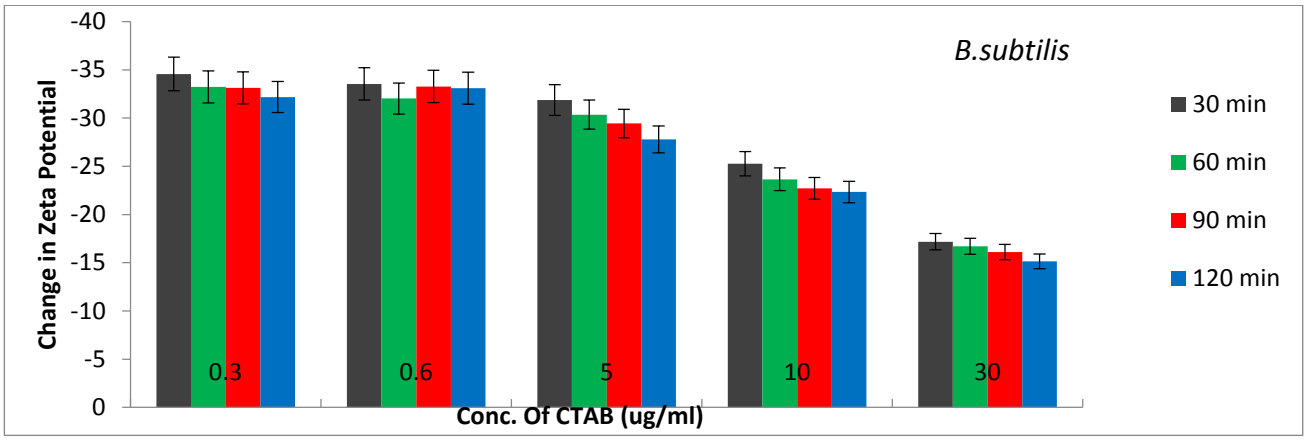


Values are expressed as mean ± S.E.M; (n = 5).

Table 1c: Effect of CTAB on the Zeta potential of the *B.subtilis* (gram positive)

Conc. Of CTAB (µg/ml) ZP of B.subtilis (-34.4)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	-34.57±1.17	-33.23±1.6	-33.133±1.6	-32.17±1.6
0.6	-33.51±1.6	-32.02±1.5	-33.275±1.6	-33.1±1.6
5	-31.84±1.5	-30.355±1.4	-29.437±1.4	-27.775±1.3
10	-25.267±1.28	-23.65±1.2	-22.7±1.2	-22.33±1.08
30	-17.187±0.8	-16.7±0.78	-16.1±0.7	-15.155±0.7

INFERENCE- CTAB causes changes in Zp of *B.subtilis* (gram positive)

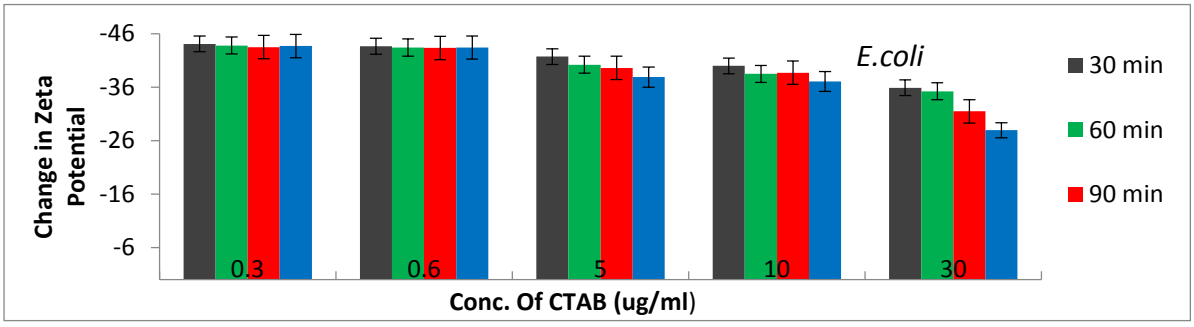


Values are expressed as mean ± S.E.M; (n = 5).

Table 1d: Effect of CTAB on the Zeta potential of the *E.coli* (gram negative)

Conc. Of CTAB (µg/ml) ZP *of E.coli* (-44.2)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	-44.1±2.20	-43.8±2.1	-43.5±2.1	-43.7±2.1
0.6	-43.67±2.1	-43.42±2.1	-43.34±2.1	-43.42±2.1
5	-41.71±2.08	-40.2±1.9	-39.6±1.8	-37.9±1.8
10	-39.98±1.9	-38.5±1.8	-38.7±1.89	-37.08±1.8
30	-35.9±1.7	-35.24±1.6	-31.48±1.4	-27.96±1.39

INFERENCE- CTAB causes changes in Zp of the *E.coli* (gram negative)

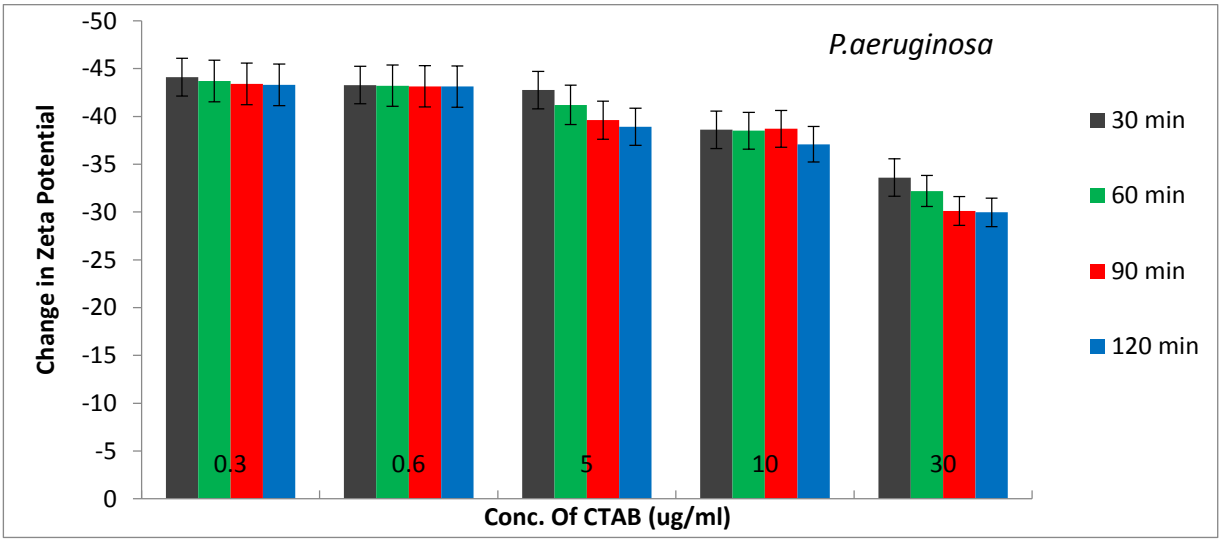


Values are expressed as mean ± S.E.M; (n = 5).

Table 1e: Effect of CTAB on the Zeta potential of the *P.aeruginosa* (gram negative)

Conc. Of CTAB (µg/ml) ZP of P.aeruginosa (-43.7)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	-44.1±2.2	-43.7±2.1	-43.4±2.1	-43.3±2.1
0.6	-43.27±2.1	-43.2±2.1	-43.14±2.1	-43.12±2.1
5	-42.76±2.0	-41.2±1.9	-39.6±1.8	-38.9±1.8
10	-38.6±1.9	-38.5±1.8	-38.7±1.8	-37.08±1.7
30	-33.6±1.6	-32.2±1.4	-30.1±1.3	-29.96±1.2

INFERENCE- CTAB causes changes in Zp *of P.aeruginosa*

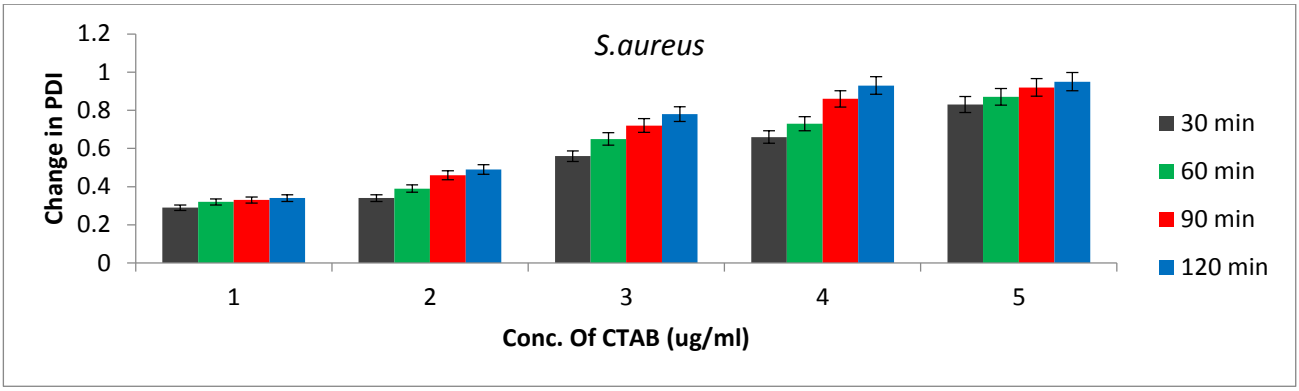


Values are expressed as mean ± S.E.M; (n = 5).

1.3. Polydispersity index measurement

Table 1f : Effect of CTAB on the PDI of the S.aureus(gram positive).

EFFECT OF CTAB ON THE PDI OF THE S.aureus (gram positive)				
PDIof S .aureus-(0.28)				
	Time of exposure			
Conc. Of CTAB (ug/ml)	30 min	60 min	90 min	120 min
0.3	0.29±0.016	0.32±0.016	0.33±0.0165	0.34±0.017
0.6	0.34±0.017	0.39±0.0195	0.46±0.0235	0.49±0.024
5	0.56±0.028	0.65±0.032	0.72±0.036	0.78±0.039
10	0.66±0.033	0.73±0.036	0.86±0.043	0.93±0.046
30	0.83±0.041	0.87±0.043	0.92±0.046	0.95±0.047
INFERENCE- CTAB causes	changes on PDI of S. aureus			



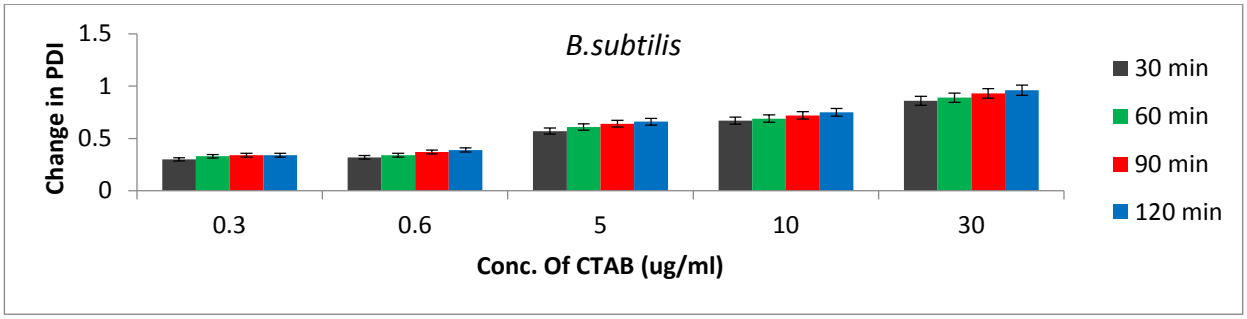
Values are expressed as mean ± S.E.M; (n = 5).

Table 1g:Effect of CTAB on the PDI OF THE *B.subtilis* (gram positive)

PDI *of B.subtilis* (0.32)

	Time of exposure			
Conc. Of CTAB (µg/ml)	30 min	60 min	90 min	120 min
0.3	0.3±0.015	0.33±0.016	0.34±0.017	0.34±0.017
0.6	0.32±0.016	0.34±0.017	0.37±0.018	0.39±0.0195
5	0.57±0.028	0.61±0.0305	0.64±0.032	0.66±0.033
10	0.67±0.033	0.69±0.035	0.72±0.036	0.75±0.033
30	0.86±0.043	0.89±0.0445	0.93±0.046	0.96±**0.048**

INFERENCE- CTAB causes changes in PDI of *B.substilis*



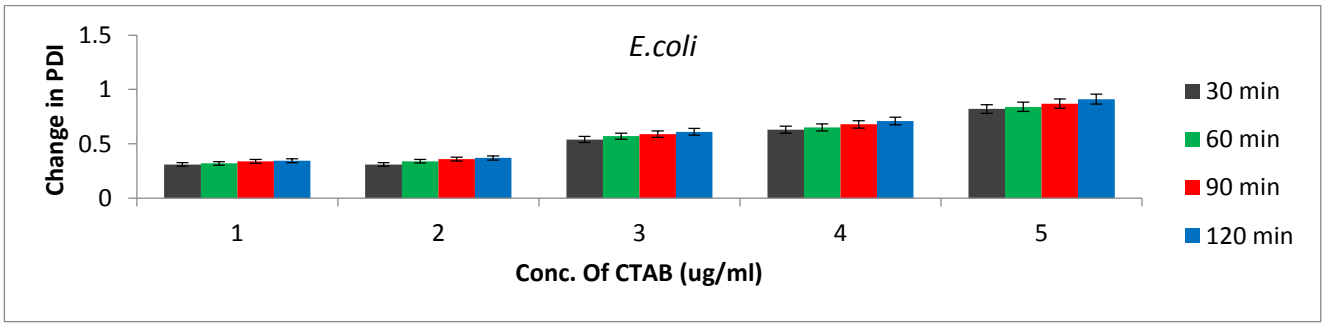
Values are expressed as mean ± S.E.M; (n = 5).

Table 1h: EFFECT OF CTAB ON THE PDI OF THE *E.coli* (gram negative)

PDI of *E.coli*- (0.31)

Conc. Of CTAB (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	0.31±0.014	0.32±0.016	0.338±0.0165	0.345±0.017
0.6	0.31±0.015	0.34±0.017	0.36±0.018	0.37±0.018
5	0.54±0.027	0.57±0.028	0.59±0.0295	0.61±0.030
10	0.63±0.031	0.65±0.032	0.68±0.038	0.71±0.035
30	0.82±0.041	0.84±0.042	0.87±0.0435	0.91±0.0455

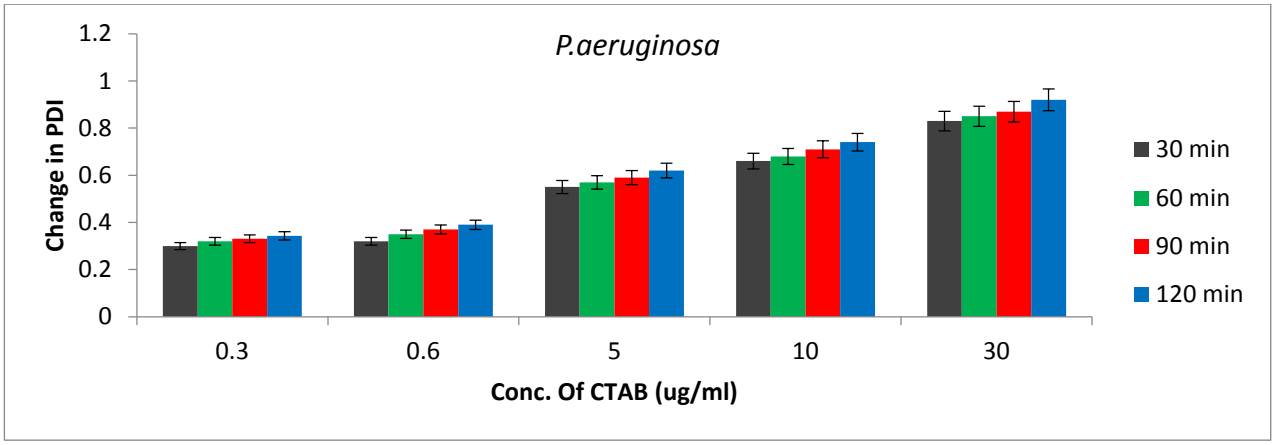
INFERENCE- CTAB cause changes in PDI of THE *E.coli* (gram negative)



Values are expressed as mean ± S.E.M; (n = 5).

Table 1I:Effect of CTAB on the PDI of the *P.aeruginosa*(gram negative)

EFFECT OF CTAB ON THE PDI OF THE P.aeruginosa (gram negative)				
PDI of P.aeruginosa (0.3)				
Conc. Of CTAB (ug/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	0.3±0.015	0.32±0.016	0.331±0.017	0.343±0.017
0.6	0.32±0.016	0.35±0.0175	0.37±0.018	0.39±0.00195
5	0.55±0.028	0.57±0.028	0.59±0.029	0.62±0.031
10	0.66±0.032	0.68±0.038	0.71±0.035	0.74±0.038
30	0.83±0.042	0.85±0.0043	0.87±-.0435	0.92±0.046
INFERENCE- CTAB causes	change in PDI of P.aeruginosa			



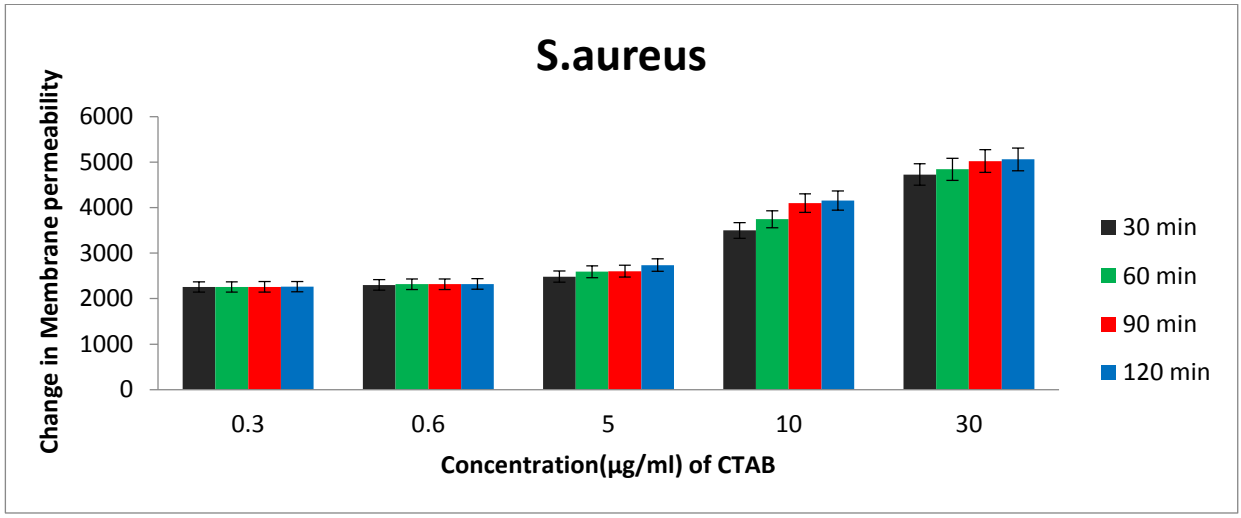
Values are expressed as mean ± S.E.M; (n = 5).

1.4. Membrane permeability measurement

Table 1J: Effect of CTB on the membrane permeability of the *S.aureus* (gram positive) S.aureus (2250.632)

Conc. Of CTAB (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	2255.874±112.23	2257.892±112.23	2259.122±112.34	2260.765±112.85
0.6	2301.324±115	2315.868±115.1	2317.439±114.32	2320.991±115.32
5	2481.83±122.43	2592.5±128.34	2601.5±129.32	2735.5±134.5
10	3497.83±172.34	3744.33±184.3	4099.1±204.34	4154.5±206.65
30	4727±235.23	4843.43±239.76	5021.83±248.43	5058.7±248.45

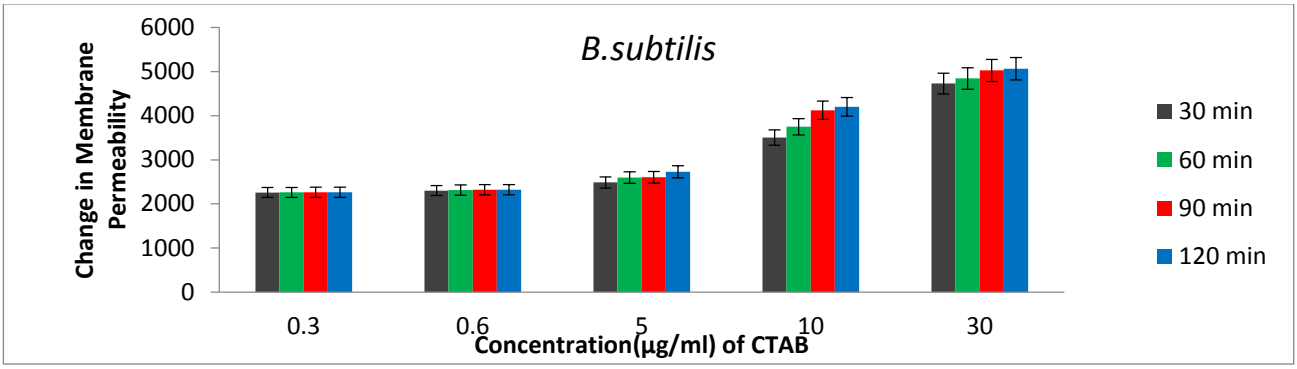
INFERENCE- CTAB cause changes in Permeability of .aureus



Values are expressed as mean ± S.E.M; (n = 5).

Table 1k :Effect of the CTAB on the membrane permeability of *the B.subtilis*(gram positive)

EFFECT OF CTAB ON THE membrane permeability OF THE B.substilis (gram positive)				
B.subtilis (2257.532)				
	Time of exposure			
Conc. Of CTAB (ug/ml)	30 min	60 min	90 min	120 min
0.3	2257.632±112.79	2260.5±112.67	2263.3±113.16	2263.7±113.45
0.6	2303.4±115.066	2317.4±115.87	2319.7±115.87	2323.1±116.15
5	2485.3±124.84	2597.9±129.21	2603.5±130.23	2730±136.54
10	3503.3±174.54	3749.5±187.54	4124±206.3	4203.2±210.15
30	4729.7±236.36	4846.2±241.9	5028.45±251.42	5067.2±253.34
INFERENCE- CTAB cause changes in Permeability		of B. subtilis		



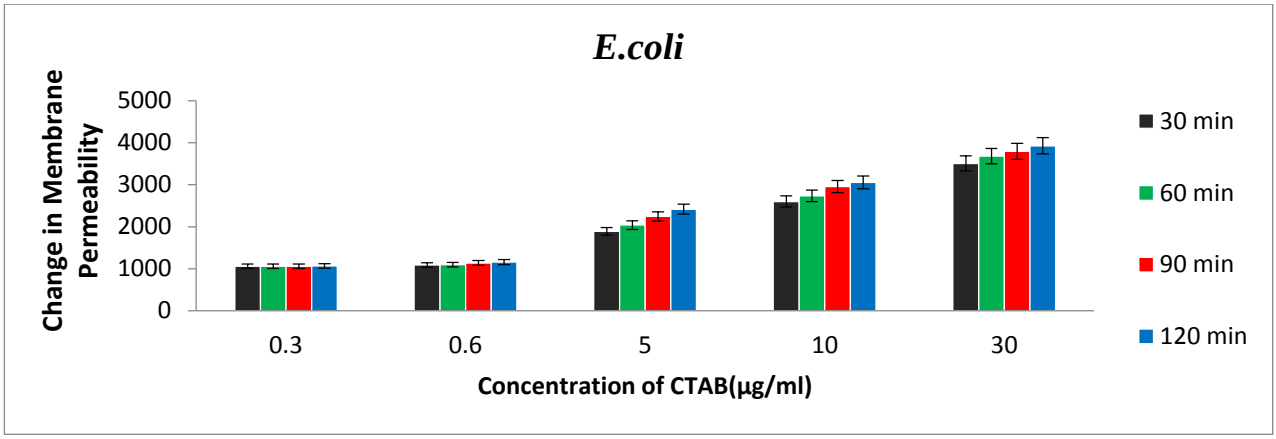
Values are expressed as mean ± S.E.M; (n = 5).

Table 1l: Effect of CTAB on the membrane permeability of the *E.coli* (gram negative)

E.coli-(1058.7)

Conc. Of CTAB (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	1059.5± 52.564	1061.37±53.05	1063.16±53.15	1067.65±53.2
0.6	1089.5±53.92	1097.61±54.5	1138.67±56.34	1159.65±57.96
5	1890.3±91.178	2039.8±101.88	2245.97±112.25	2419.1±120.94
10	2602.5±129.8	2736.17±136.78	2956.78±147.8	3052.8±152.5
30	3508.8±174.6	3679.2±183.34	3795.72±189.75	3926.2±196.3

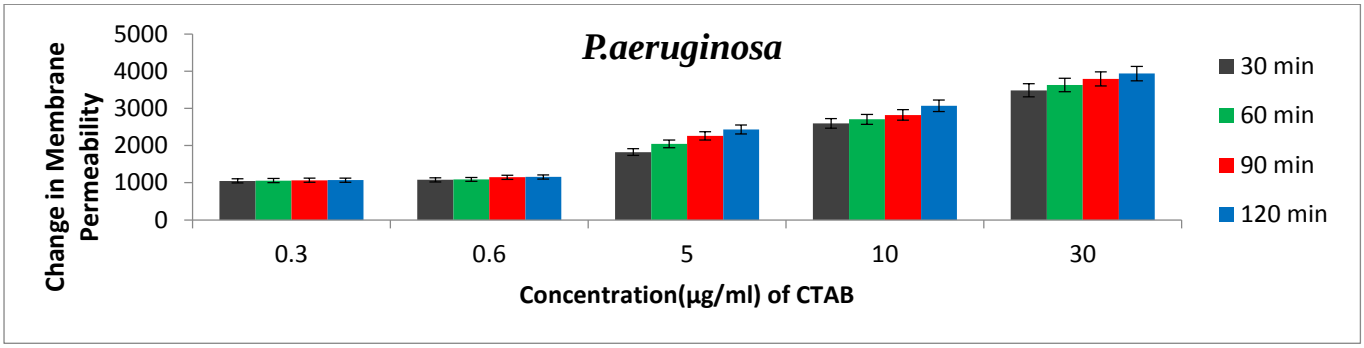
INFERENCE- CTAB causes changes in Permeability of the *E.coli* (gram negative)



Values are expressed as mean ± S.E.M; (n = 5).

Table 1m:Effect of CTAB on the membrane permeability of the *P.aeruginosa* (gram negative)

EFFECT OF CTAB ON THE membrane permeability OF THE P.aeruginosa (gram negative)				
P.aeruginosa 1050.4				
	Time of exposure			
Conc. Of CTAB (ug/ml)	30 min	60 min	90 min	120 min
0.3	1051.2±50.32	1059.34±52.67	1067.7±54.34	1068.5±53.7
0.6	1078.2±51.34	1089.4±53.23	1147.67±54.32	1156.78±56.21
5	1823.56±86.4	2045.8±0..52	2260.3±116.22	2430.43±123.9
10	2598.3±127.34	2705.4±132.23	2821.45±133.32	3067.57±158.4
30	3486±170.34	3624.9±180.3	3789.6±190.6	3934.3±198.2
INFERENCE- CTAB causes changes in Permeability of P.aeruginosa				



Values are expressed as mean ± S.E.M; (n = 5).

2. Effect of Polymixin B on *S. aureus, B. Subtilis, E.coli and P. aeruginosa*

2.1.Minimum inihibitory concentration

Table2a. Minimum inihibitory concentration (MIC; µg/ml) of polymixin B against gram positive and gram negative organisms.

Agents	organisms	Concentration(µg/ml)
Polymixin B	*S.aureus*	0.25
	B.subtilis	0.25
	E.coli	3.125

	P.aeruginosa	3.125

2.2. Zeta potential measurement:

Table 2b : Effect of PolymixinB on the ZETA POTENTIAL of the *S.aureus* and *E.coli* (gram positive)
ZP effect of Polymixin B on E.coli and S.aureus at diff conc. at diff time interval.

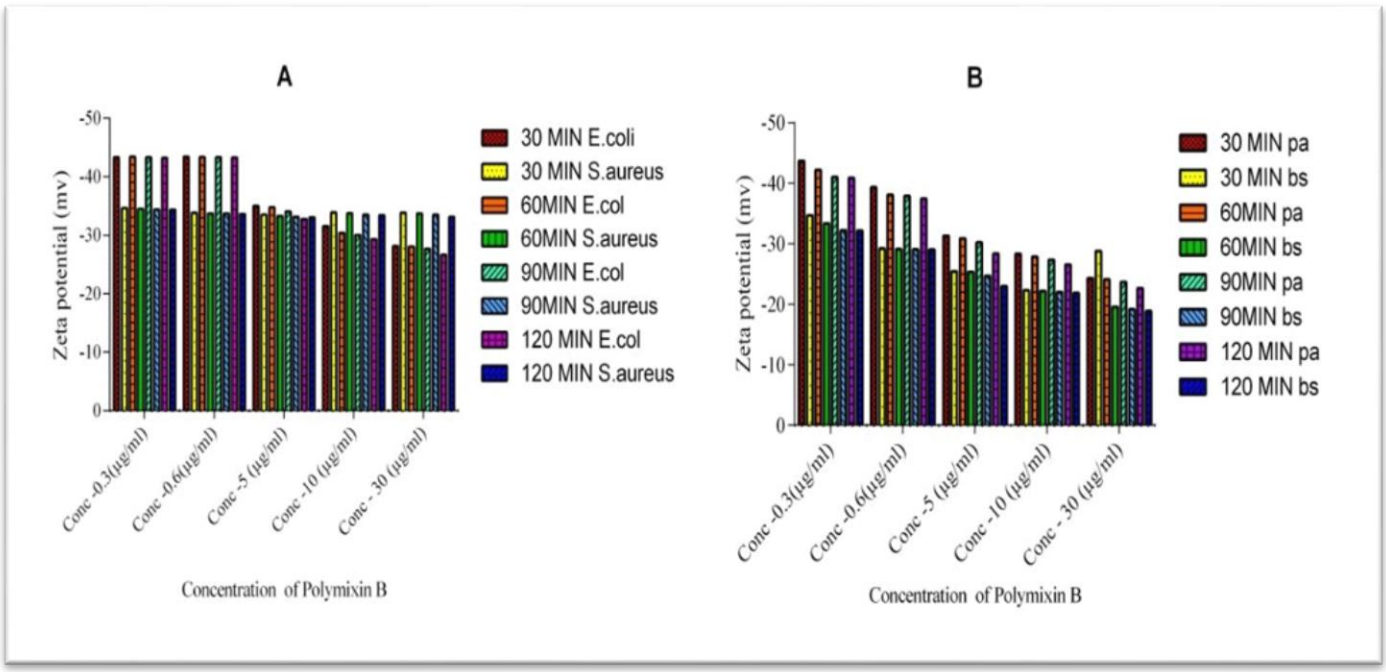
Time	30 MIN		60MIN		90MIN		120 MIN	
Concentration	E.coli	S.aureus	E.coli	S.aureus	E.coli	S.aureus	E.coli	S.aureus
Conc -0.3(µg/ml)	-43.3	-34.63	-43.43	-34.53	-43.33	-34.375	-43.23	-34.37
Conc -0.6(µg/ml)	-43.45	-33.865	-43.35	-33.73	-43.33	-33.73	-43.275	-33.63
Conc -5 (µg/ml)	-35.012	-33.54	-34.7511	-33.28	-34.07	-33.16	-32.76	-33.081
Conc -10 (µg/ml)	-31.551	-33.901	-30.41	-33.769	-30.08	-33.508	-29.33	-33.438
Conc - 30 (µg/ml)	-28.15	-33.865	-28.05	-33.73	-27.67	-33.53	-26.65	-33.16

Inference: Polymixin B causes very little changes on ZP (conc. and time dependent) in presence of both *E.coli* (gram negative) and *S.aureus* (gram positive) bacteria.

Table 2c: ZP effect of Polymixin B on *P.aeruginosa* and *B.subtilis* at diff conc. at diff time interval

Time	30 MIN		60MIN		90MIN		120 MIN	
Concentration	P.aureus	B. subtilis	P. aureus	B. subtilis	P. aureus	B. subtilis	P. aureus	B. subtilis
Conc -0.3(µg/ml)	-43.7	-34.67	-42.2	-33.34	-41.02	-32.23	-40.87	-32.13
Conc -0.6(µg/ml)	-39.32	-29.18	-38.06	-29.12	-37.87	-29.07	-37.45	-29
Conc -5 (µg/ml)	-31.3	-25.43	-30.87	-25.3	-30.23	-24.67	-28.34	-22.96
Conc -10 (µg/ml)	-28.3	-22.3	-27.87	-22.2	-27.34	-22	-26.56	-21.87
Conc - 30 (µg/ml)	-24.32	-28.8	-24.1	-19.56	-23.7	-19.2	-22.65	-18.9

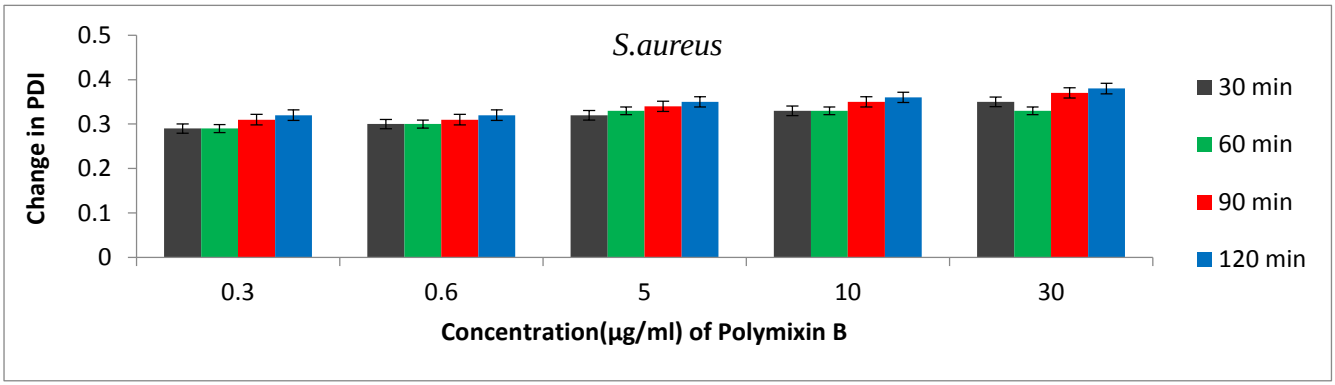
Inference: Polymixin B causes very little changes on ZP (conc. and time dependent) in presence of both *P.aeruginosa* (gram negative) and *B. subtilis* (gram positive) bacteria.



2.3.Polydispersity index measurement

Table 2d:Effect of polymixin B on the PDI of the *S.aureus* (gram positive).

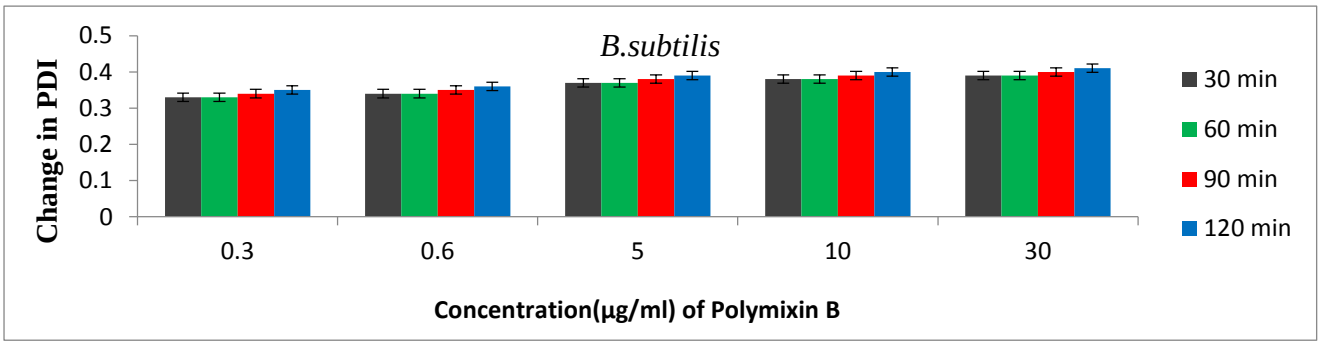
EFFECT OF polimyxin B ON THE PDI OF THE THE S . aureus (gram positive)				
PDI of S. aureus 0.28				
	Time of exposure			
Conc. Of Polymixin B(ug/ml)	30 min	60 min	90 min	120 min
0.3	0.29±0.0145	0.29±0.014	0.31±0.0155	0.32±0.016
0.6	0.3±0.015	0.3±0.015	0.31±0.0155	0.32±0.016
5	0.32±0.016	0.33±0.016	0.34±0.0170	0.35±0.017
10	0.33±0.0165	0.33±0.016	0.35±0.017	0.36±0.0175
30	0.35±0.0175	0.33±0.016	0.37±0.017	0.38±0.018
INFERENCE- Polymixin B causes no changes in PDI of TH E s.a		PDI of the S. aureus		



Values are expressed as mean ± S.E.M; (n = 5).

Table 2e:Effect of Polymixin B on the PDI of the *B,subtilis*(gram positive)

EFFECT OF Polimyxin B ON THE PDI OF THE B.subtilis (gram possitive)				
PDI of B. subtilis 0.32				
	Time of exposure			
Conc. Of Polymixin B (ug/ml)	30 min	60 min	90 min	120 min
0.3	0.33±0.016	0.33±0.016	0.34±0.017	0.35±0.017
0.6	0.34±0.0165	0.34±0.016	0.35±0.017	0.36±0.017
5	0.37±0.017	0.37±0.017	0.38±0.018	0.39±0.019
10	0.38±0.18	0.38±0.018	0.39±0.018	0.4±0.019
30	0.39±0.018	0.39±0.18	0.4±0.019	0.41±0.0195
INFERENCE- polymixin B causes	no changes in PDI of the B. subtilis			



Values are expressed as mean ± S.E.M; (n = 5).

Table 2f:Effect of Polymixin B on the PDI of the E.Coli(gram negative)

EFFECT OF polimyxin B ON THE PDI OF THE THE E.coli (gram negative)				
PDIof e . Coli 0.31				
	Time of exposure			
Conc. Of Polymixin B (ug/ml)	30 min	60 min	90 min	120 min
0.3	0.32±0.016	0.36±0.017	0.39±0.018	0.45±0.024
0.6	0.34±0.16	0.38±0.018	0.42±0.021	0.46±0.025
5	0.54±0.027	0.57±0.028	0.59±0.29	0.63±0.31
10	0.63±0.31	0.65±0.029	0.68±0.29	0.71±0.035
30	0.72±0.36	0.74±0.037	0.77±0.038	0.82±0.041
INFERENCE- Polymixin B causes changes in PDI of THE E.coli (gram negative)				

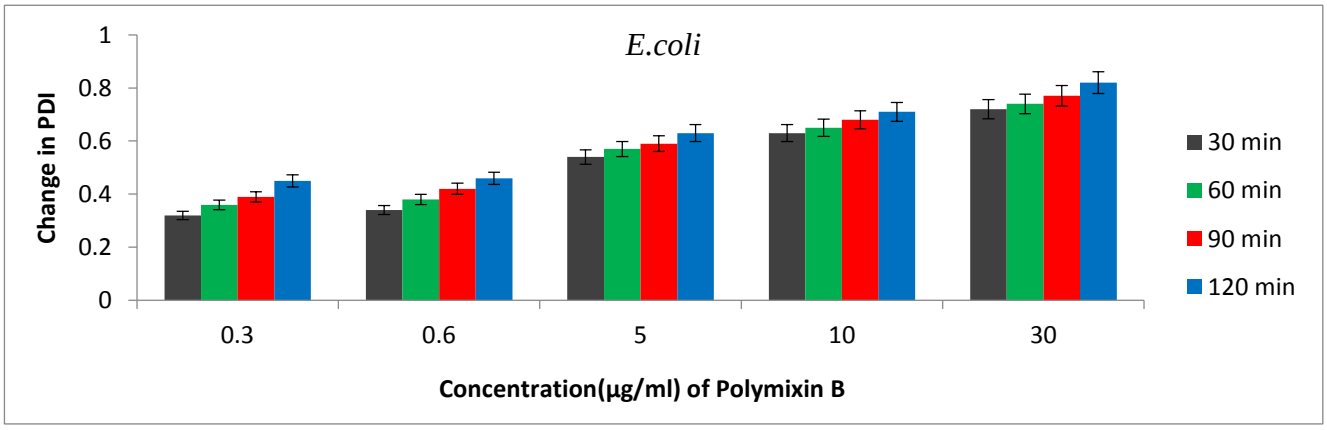
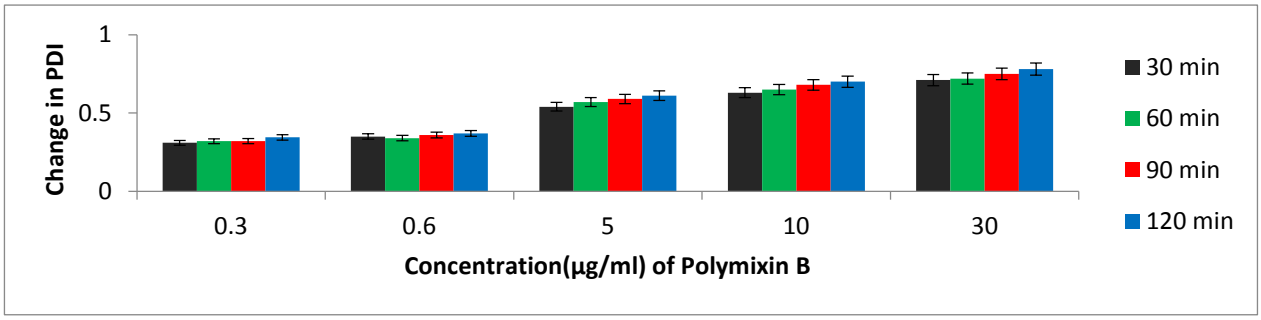


Table 2g:Effect of Polimyxin B on the PDI of the P.aeruginosa (gram negative)

PDI of P. aeruginosa 0.3

Conc. Of Polymixin B (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	0.31±0.016	0.32±0.016	0.321±0.016	0.345±0.016
0.6	0.35±0.017	0.34±0.016	0.36±0.018	0.37±0.017
5	0.54±0.027	0.57±0.0028	0.59±0.029	0.61±0.031
10	0.63±0.031	0.65±0.029	0.68±0.029	0.7±0.0036
30	0.71±0.0036	0.72±0.00365	0.75±0.037	0.781±0.0387

INFERENCE- CTAB causes changes in PDI of THE P.aeruginosa (gram negative)



Values are expressed as mean ± S.E.M; (n = 5).

2.4.Membrane permeability on gram positive and gram negative bacteria.

Table 2h :Membrane permeability effect of Polymixin B *on E.coli* and *S.aureus* at diff conc. at diff time interval

Concentration	*E.coli*	*S.aureus*	*E.coli*	*S.aureus*	*E.coli*	*S.aureus*	*E.coli*	*S.aureus*
Conc -0.3(µg/ml)	988.83±49.44	983.5±49.17	990.5±49.5	984.67±49.3	991.83±49.55	985.83±49.25	993.17±49.65	986.83±49.3
Conc -0.6(µg/ml)	1001.5±50.05	986.21±49.34	1007.7±50.35	988.5±49.4	1010.5±50.5	989.5±49.45	1111.7±55.5	990.029±49.5
Conc -5(µg/ml)	1532.87±76.6	995.98±49.75	1545.63±77.25	1008.31±50.4	1555.09±77.75	1009.69±50.5	1599.87±79.8	1011.65±50.54
Conc -10(µg/ml)	1647.06±82.35	1001.5±50.05	1665.76±83.25	1009.17±50.45	1688.4±84.4	1013.7±50.65	1699.43±84.9	1022.09±51.1
Conc -30(µg/ml)	1769.67±88.45	1013.5±50.65	1776.5±88.8	1015.17±50.75	1784.5±89.3	1020.33±51	1794.7±89.7	1033±51.65

Inference: Polymixin B causes changes on membrane permeability (conc. and time dependent) in presence of both *E.coli* (gram negative) and *S.aureus* (gram positive) bacteria.

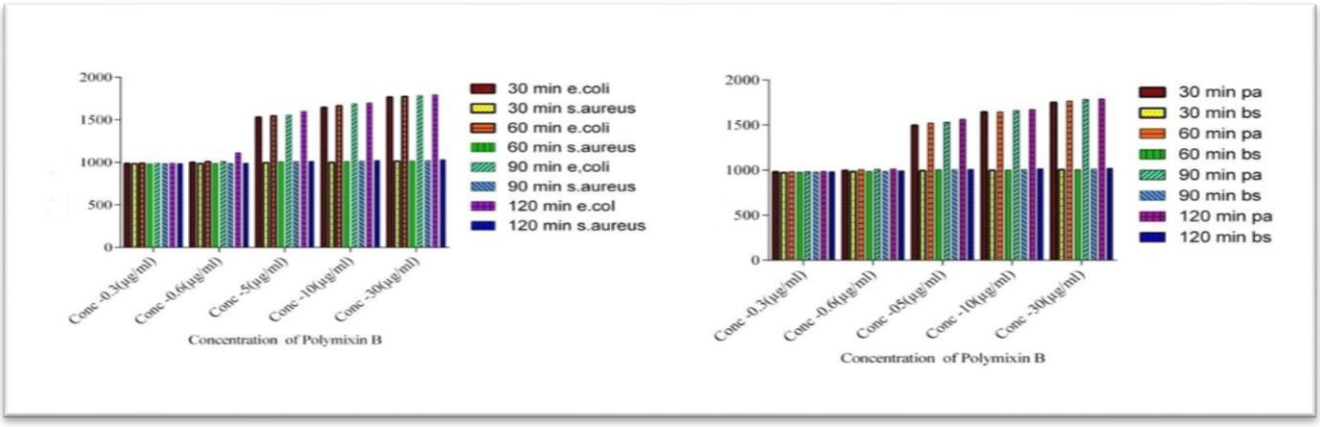


Table 2i: Membrane permeability effect of Polymixin B on P.aeruginosa and B.subtilis at diff conc.at different time interval

Time	30 min		60 min		90 min		120 min	
Concentration	*P. aureus*	*B. subtilis*	*P.aureus*	*B. subtilis*	*P. aureus*	*B. subtilis*	*P.aureuus*	*b.subtilis*
Conc -0.3(µg/ml)	980.5±49	973.31±48.65	982.45±49.9	975.34±48.75	983.56±49.15	978.4±47.4	985.23±49.25	980.3±49
Conc -0.6(µg/ml)	995.87±49.75	983.5±49.13	1004.78±50.04	985.6±49.25	1008.32±50.4	987.3±49.35	1010.3±50.5	990.4±49.5
Conc -05(µg/ml)	1498.74±74.93	993.4±49.64	1523.98±76.15	1003.64±50.15	1532.4±76.15	1005.5±50.25	1565.3±78.6	1007.4±50.35
Conc -10(µg/ml)	1645.43±82.25	997.48±49.87	1643.83±82.15	1000±50	1659.8±82.95	1008.2±50.4	1672.4±83.8	1015.7±50.75
Conc -30(µg/ml)	1752.58±87.5	1006.35±50.3	1768.3±88.4	1004.3±50.2	1783.4±89.15	1012.2±50.6	1789.7±89.45	1021.6±51.05

Inference: Polymixin B causes changes on membrane permeability (conc. and time dependent) in presence of both P.aeruginosa (gram negative) and B. subtilis (gram positive) bacteria.

3.Effect of Daptomycin on S. aureus, B. Subtilis, E.coli and P. aeruginosa

3.1.Minimum inihibitory concentration

Table3a. Minimum inihibitory concentration (MIC) of Daptomycin against gram positive and gram negative organisms

Agents	Organisms studied	Concentration(µg/ml)
Daptomycin	*S.aureus*	0.625
	B.subtilis	0.312
	E.coli	0.25
	P.aeruginosa	0.25

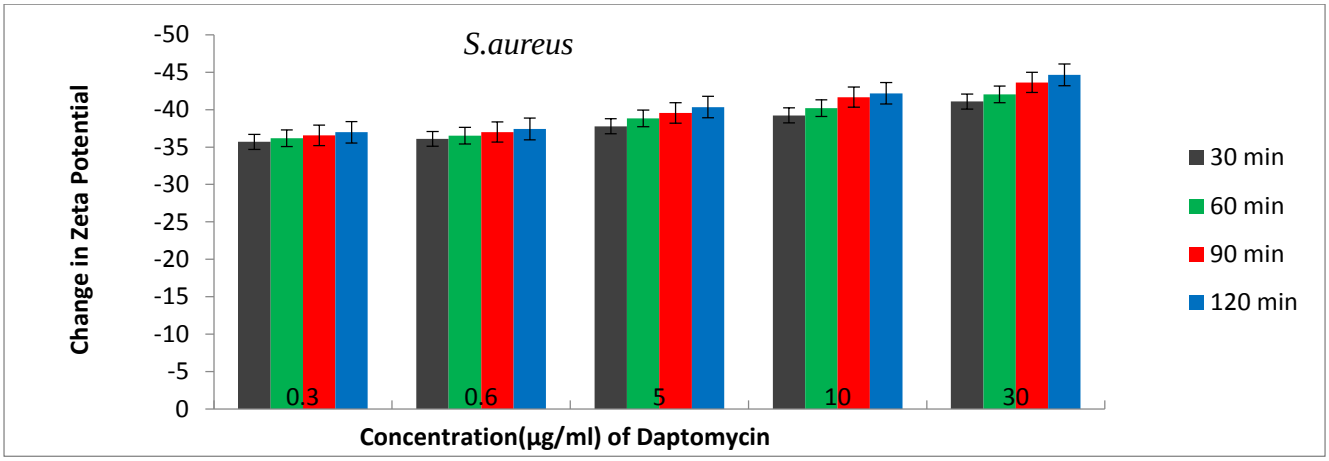
3.2.Zeta Potential (ZP) measurement

Table3b : Effect OF Daptomycin on the ZETA POTENTIAL of the S.*aureus* (gram positive)

ZP of S aureus -35.6

Conc. Of Daptomycin (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	-35.69±1.7	-36.17±1.7	-36.56±1.76	-36.98±1.7
0.6	-36.09±1.78	-36.51±1.7	-37±1.8	-37.42±1.8
5	-37.78±1.8	-38.84±1.9	-39.56±1.9	-40.34±1.9
10	-39.23±1.9	-40.21±1.9	-41.67±2.05	-42.18±2.1
30	-41.09±2.06	-42.03±2.1	-43.63±2.1	-44.64±2.1

INFERENCE- Daptomycin causes changes in ZP of *S.aureus*



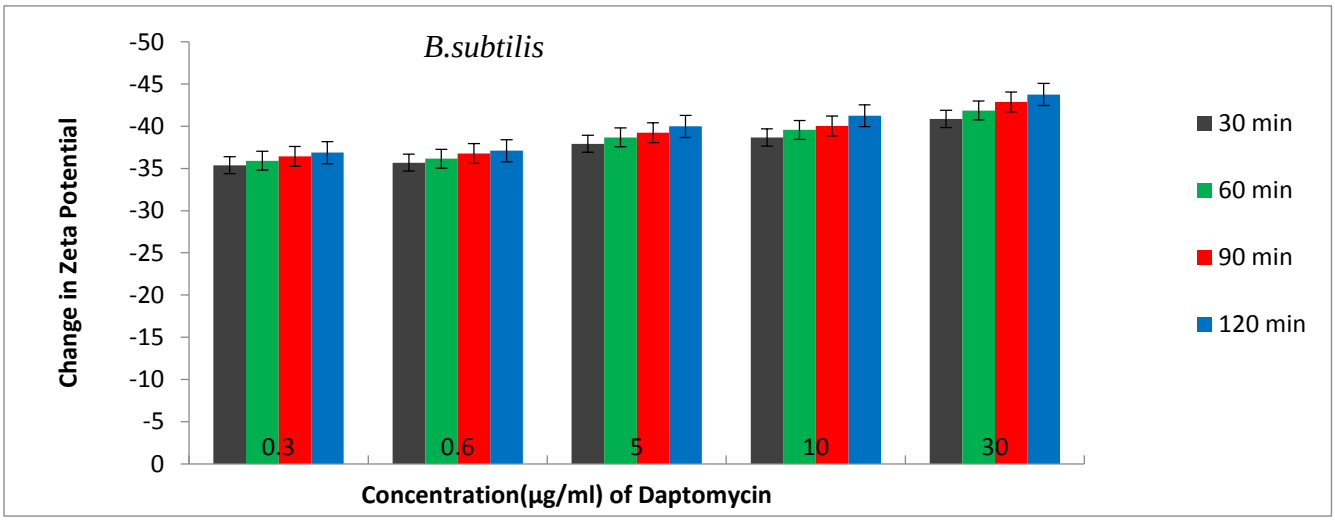
Values are expressed as mean ± S.E.M; (n = 5).

Table 3c:EFFECT OF Daptomycin ON THE ZETA POTENTIAL OF THE B.substilis (gram positive)

ZP of S .aureus -35.3

Conc. Of Daptomycin (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	-35.38±1.7	-35.91±1.7	-36.43±1.78	-36.87±1.7
0.6	-35.68±1.7	-36.15±1.78	-36.76±1.7	-37.1±1.85
5	-37.92±1.89	-38.68±1.9	-39.23±1.9	-39.98±1.9
10	-38.67±1.9	-39.56±1.9	-40.02±1.98	-41.24±2.06
30	-40.87±1.98	-41.87±2.07	-42.89±2.14	-43.76±2.1

INFERENCE-Daptomycin causes changes in Zp of *B.subtilis*



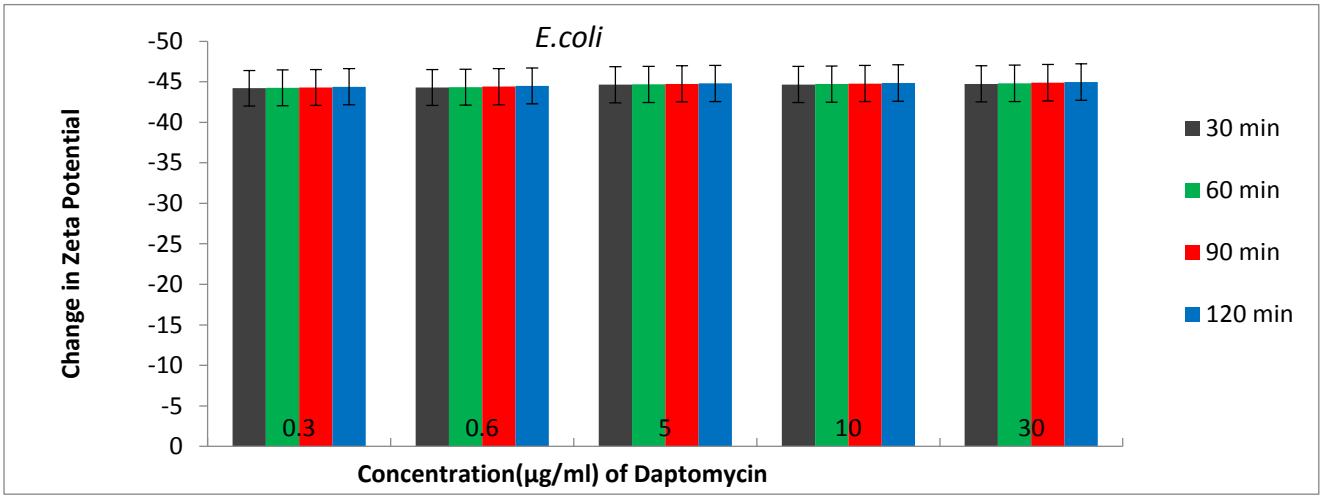
Values are expressed as mean ± S.E.M; (n = 5).

Table 3d:EFFECT OF Daptomycin on the ZETA POTENTIAL of the E.coli (gram negative)

ZP of E. coli -44.2

Conc. Of Daptomycin (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	-44.2±2.2	-44.25±2.2	-44.3±2.2	-44.39±2.2
0.6	-44.3±2.2	-44.33±2.2	-44.4±2.2	-44.49±2.2
5	-44.65±2.2	-44.69±2.2	-44.74±2.2	-44.8±2.2
10	-44.67±2.2	-44.72±2.2	-44.79±2.2	-44.86±2.2
30	-44.75±2.2	-44.81±2.2	-44.88±2.2	-44.98±2.2

INFERENCE- Daptomycin causes no changes in zp of the E.coli (gram negative)



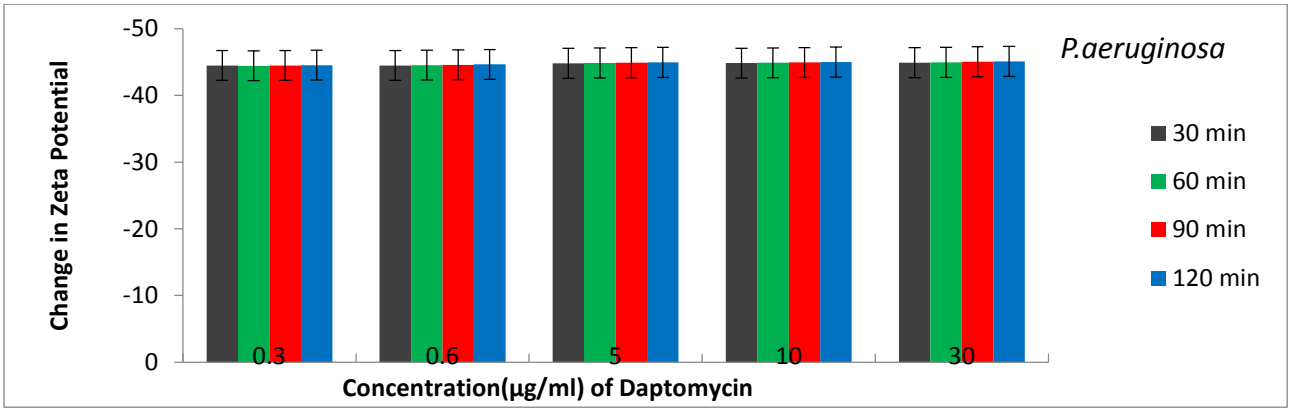
Values are expressed as mean ± S.E.M; (n = 5).

Table 3e:Effect of Daptomycin on the ZETA POTENTIAL on the P. aeruginosa (gram negative)

ZP of P .aeruginosa - 44.4

Conc. Of Daptomycin (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	-44.5±2.26	-44.44±2.2	-44.49±2.2	-44.54±2.2
0.6	-44.5±2.2	-44.54±2.2	-44.59±2.2	-44.65±2.2
5	-44.81±2.2	-44.86±2.2	-44.91±2.2	-44.96±2.2
10	-44.84±2.2	-44.89±2.2	-4.49E+01±2.2	-44.99±2.2
30	-44.92±2.2	-44.97±2.2	-45.04±2.2	-45.09±2.2

INFERENCE- Daptomycin causes no changes in Zp of P.aeruginosa



Values are expressed as mean ± S.E.M; (n = 5).

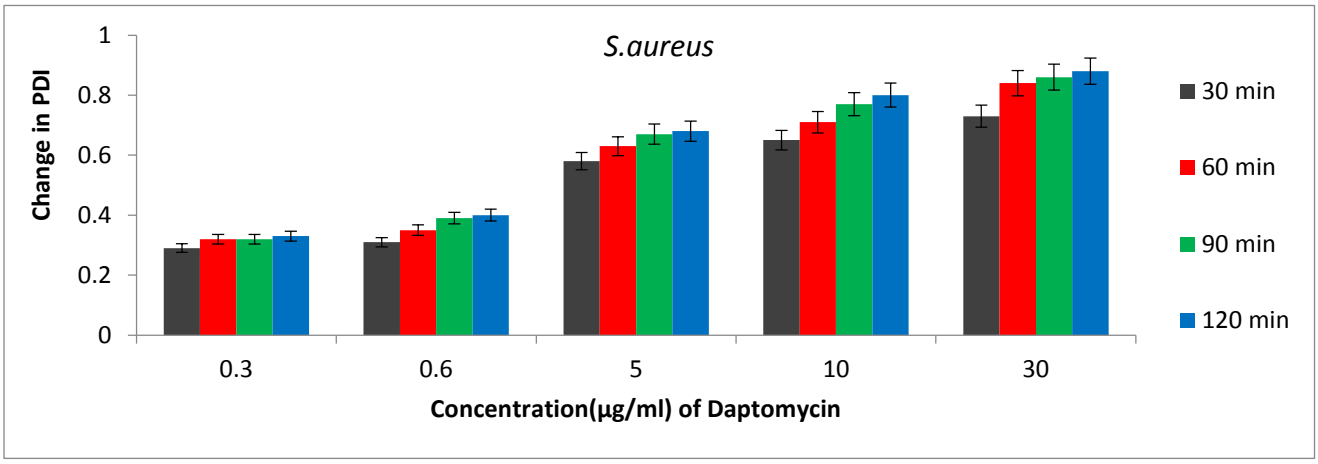
3.3.Effect of Daptomycin on Polydispersity index(PDI) on gram positive and gram negative bacteria

Table 3g:EFFECT OF Daptomycin ON THE PDI OF THE S.aureus (gram positive)

PDI of S. aureus- (0.28)

Conc. Of Daptomycin(µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	0.29±0.0145	0.32±0.016	0.32±0.016	0.33±0.0165
0.6	0.31±0.0155	0.35±0.0175	0.39±0.0195	0.4±0.020
5	0.58±0.029	0.63±0.0315	0.67±0.0325	0.68±0.034
10	0.65±0.0325	0.71±0.0355	0.77±0.0375	0.8±0.0390
30	0.73±0.0365	0.84±0.042	0.86±0.0420	0.88±0.042

INFERENCE- Daptomycin causes changes in PDI of *S.aureus*



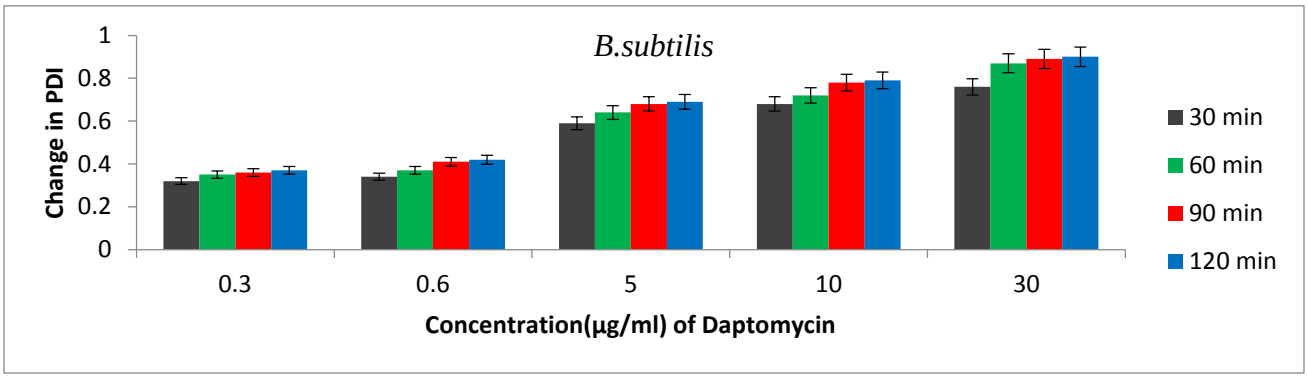
Values are expressed as mean ± S.E.M; (n = 5).

Table 3h:EFFECT of Daptomycin ON THE PDI OF THE *B.substilis* (gram positive)

PDI of B. subtilis (0.32)

Conc. Of Daptomycin (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	0.32±0.016	0.35±0.00175	0.36±0.0180	0.37±0.0185
0.6	0.34±0.170	0.37±0.018	0.41±0.0205	0.42±0.021
5	0.59±0.0295	0.64±0.0320	0.68±0.0330	0.69±0.0345
10	0.68±0.034	0.72±0.0360	0.78±0.038	0.79±0.0385
30	0.76±0.038	0.87±0.0435	0.89±0.043	0.9±0.043

INFERENCE- Daptomycin causes changes in PDI of *B.substilis*



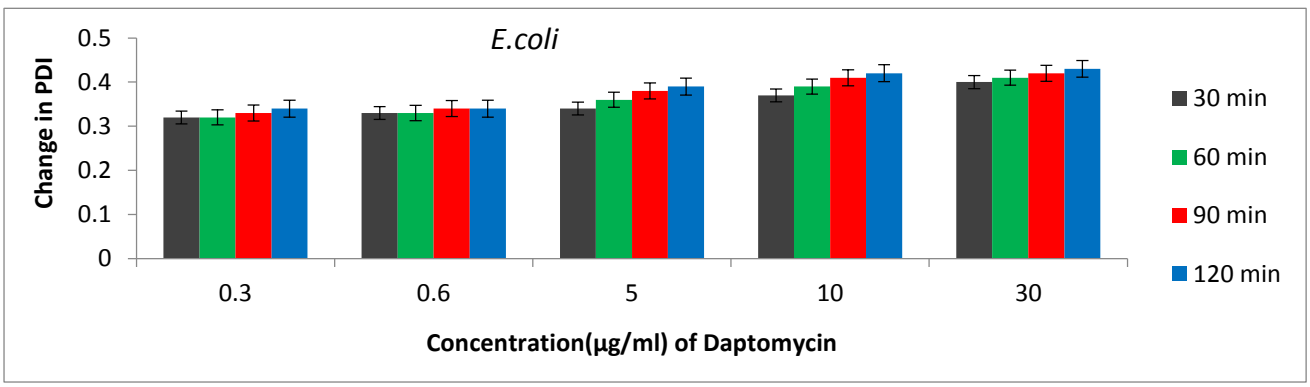
Values are expressed as mean ± S.E.M; (n = 5).

Table 3i:Effect of Daptomycin on the PDI of the *E.coli* (gram negative)

PDI of E. coli (0.32)

Conc. Of Daptomycin (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	0.32±0.0160	0.32±0.016	0.33±0.016	0.34±0.0170
0.6	0.33±0.0164	0.33±0.0165	0.34±0.0170	0.34±0.0170
5	0.34±0.0170	0.36±0.0180	0.38±0.0175	0.39±0.0190
10	0.37±0.0175	0.39±0.0190	0.41±0.0205	0.42±0.0210
30	0.4±0.02	0.41±0.0205	0.42±0.0210	0.43±0.0215

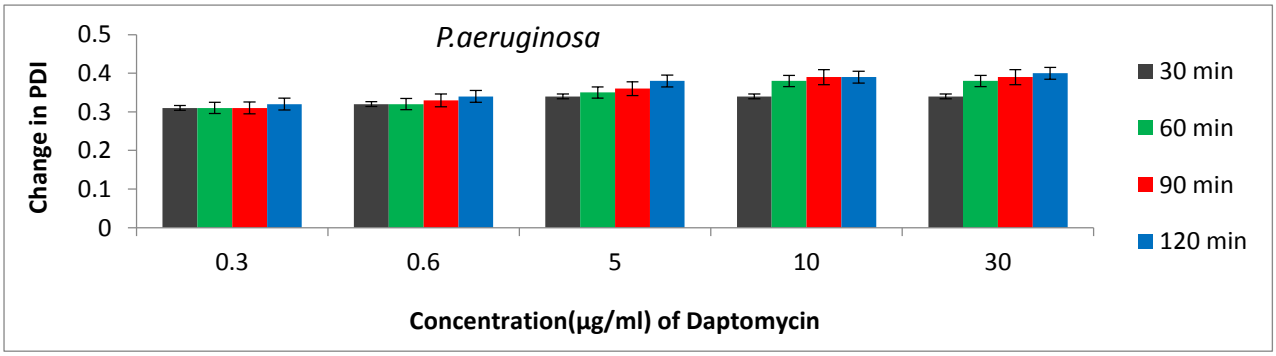
INFERENCE- Daptomycin causes no changes in PDI of THE *E.coli* (gram negative)



Values are expressed as mean ± S.E.M; (n = 5).

Table 3j:Effect of Daptomycin on the PDI of the *P.aeruginosa*(gram negative)

EFFECT OF Daptomycin ON THE PDI OF THE p.aeruginosa (gram negative)				
PDI of P .aeruginosa (0.31)				
	Time of exposure			
Conc. Of Daptomycin (ug/ml)	30 min	60 min	90 min	120 min
0.3	0.31±o.0155	0.31±0.015	0.31±0.0155	0.32±0.0160
0.6	0.32±0.0.016	0.32±0.016	0.33±0.0164	0.34±0.0170
5	0.34±0.017	0.35±0.0175	0.36±0.0180	0.38±0.018
10	0.34±0.017	0.38±0.018	0.39±0.0190	0.39±0.019
30	0.34±0.017	0.38±0.018	0.39±0.0190	0.4±0.02
INFERENCE- Daptomycin causes no changes		in PDI of P. aeruginoa		(gram negetive)



Values are expressed as mean ± S.E.M; (n = 5).

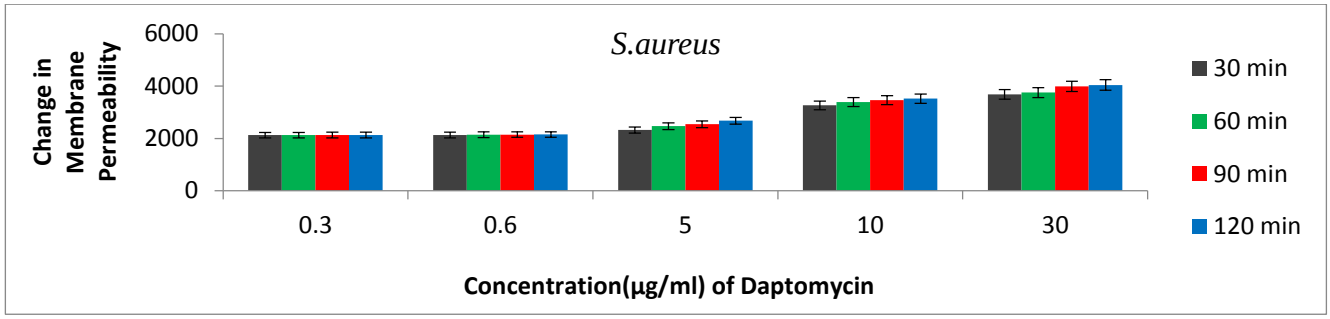
3.4.Effect of Daptomycin on the NPN uptake

Table 3k :Effect of Daptomycin on the membrane permeability of *S.aureus* (gram positive)

S aureus (2122.51)

Conc. Of Daptomycin (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	2125.4±106.25	2127.5±106.34	2129.7±106.45	2130.2±106.5
0.6	2131.56±106.5	2145.5±107.25	2148.45±107.4	2151.4±107.55
5	2324.67±116.2	2467.67±123.35	2543.42±127.15	2675.34±133.75
10	3265.45±163.25	3387.76±169.35	3466.78±173.3	3522.23±176.1
30	3687.67±184.35	3754.34±187.7	3987.34±199.35	4038.7±201.9

INFERENCE- Daptomycin causes changes in Permeability of *S.aureus* but less CTAB

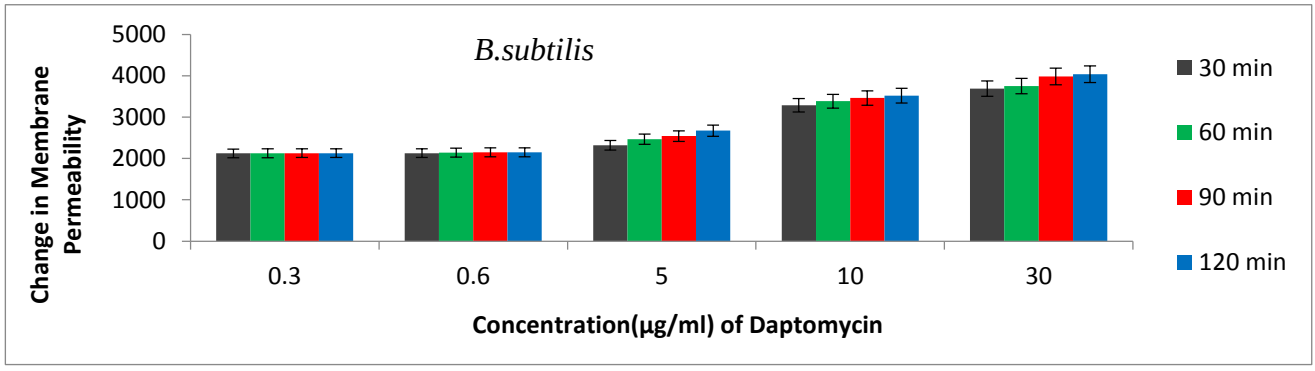


Values are expressed as mean ± S.E.M; (n = 5).

Table 3l:Effect Of Daptomycin on the membrane permeability of the *B.substilis* (gram positive)

Conc. Of Daptomycin (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	2122.456±106.12	2124.54±106.2	2127.63±106.35	2129.4±106.45
0.6	2128.73±106.36	2143.67±107.15	2147.34±107.3	2150.84±107.5
5	2321.45±116.05	2463.45±123.15	2540.23±127	2672.41±133.6
10	3287.63±164.35	3383.34±169.15	3463±173.15	3517.7±175.85
30	3688.76±184.4	3750.4±187.5	3983.23±199.14	4035.24±201.75

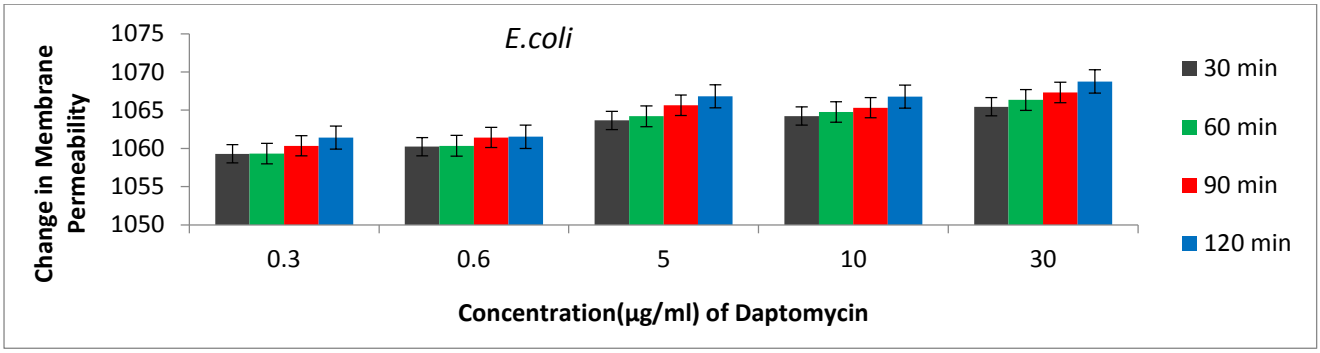
INFERENCE- Daptomycin causes changes in Permeability of B.substilis



Values are expressed as mean ± S.E.M; (n = 5).

Table 3m:Effect of Daptomycin on the membrane permeability of the E.coli(gram negative)

EFFECT OF Daptomycin ON THE membrane permeability OF THE E.coli (gram negative)				
e.c- 1058.7				
	Time of exposure			
Conc. Of Daptomycin (ug/ml)	30 min	60 min	90 min	120 min
0.3	1059.29±52.95	1059.32±52.95	1060.34±53.2	1061.4±53.27
0.6	1060.23±53.02	1060.34±53.04	1061.43±53.3	1061.52±53.36
5	1063.67±53.14	1064.2±53.2	1065.65±53.3	1066.83±53.43
10	1064.23±53.2	1064.76±53.4	1065.32±53.35	1066.76±53.45
30	1065.45±53.24	1066.34±53.3	1067.32±53.4	1068.76±53.53
INFERENCE- Daptomycin causes no changes in Permeability of THE E.coli (gram negative)				



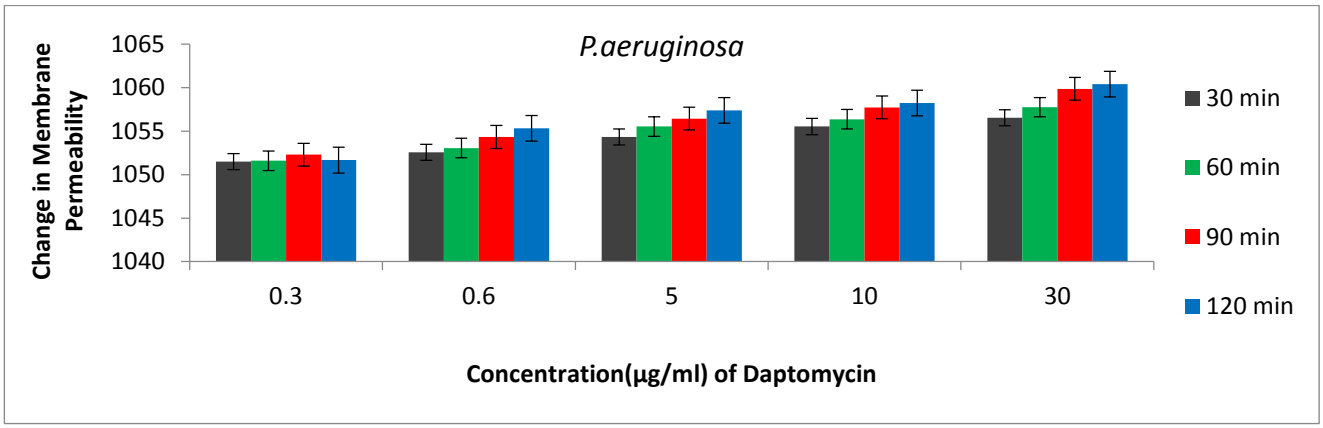
Values are expressed as mean ± S.E.M; (n = 5).

Table 3n:Effect of Daptomycin on the membrane permeability of the *P.aeruginosa* (gram negative)

P.aeruginosa 1050.4

Conc. Of Daptomycin (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	1051.5±52.55	1051.6±52.58	1052.3±52.6	1051.67±52.15
0.6	1052.56±52.59	1053.06±52.61	1054.34±52.78	1055.34±52.7
5	1054.34±52.78	1055.54±52.87	1056.45±52.9	1057.39±52.81
10	1055.54±52.87	1056.38±52.9	1057.74±52.92	1058.23±52.88
30	1056.54±52.9	1057.76±52.92	1059.87±52.97	1060.42±52.98

INFERENCE-Daptomycin causes no changes in Permeability of P.aeruginosa



Values are expressed as mean ± S.E.M; (n = 5).

II. Effect of CTAB, Polymyxin B and Daptomycin on Red blood cells.

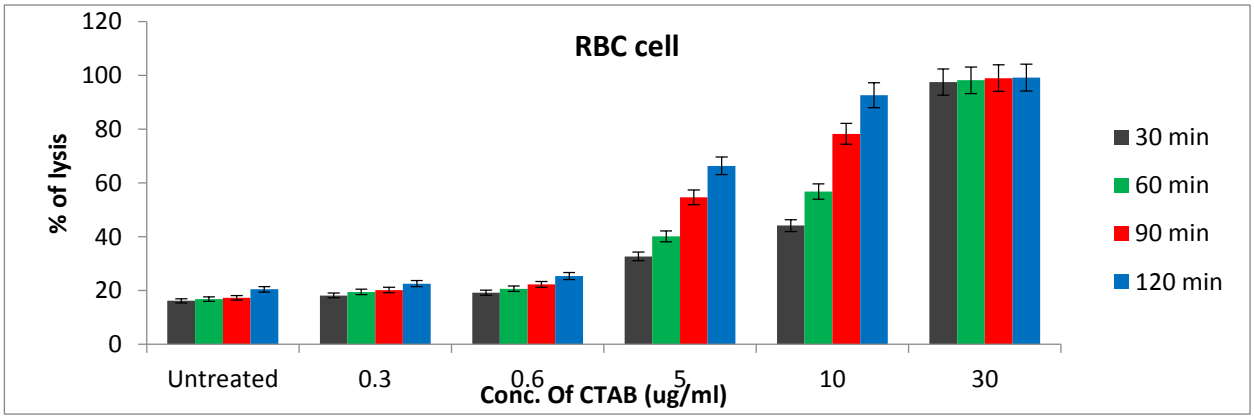
In this study we have determined the effect of these compounds on RBC the ascertaining their effects on Zeta potential (ZP), Polydispersity Index (PDI) and NPN uptake.

1. Effect of CTAB on the RBC lysis

Table 4a: Effect of CTAB on the RBC lysis (calculated as % of lysis)

Conc. of CTAB (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0	16.2 ± 1.2	16.8 ± 1.4	17.3 ±2.1	20.5 ± 2.3
0.3	18.2 ± 1.4	19.5 ± 0.8	20.2 ± 1.2	22.6 ± 1.2
0.6	19.2 ± 1.5	20.7 ± 1.1	22.3 ± 1.6	25.4 ± 1.8
5	32.7 ± 1.9	40.2 ± 1.8	54.7 ± 2.3	66.4 ± 3.2
10	44.2 ± 2.3	56.8 ± 1.9	78.3 ± 3.2	92.7 ± 2.8
30	97.5 ± 2	98.2 ± 1.2	99 ± 0.5	99.2 ± 0.5

Inference- CTAB causes RBC lysis



Values are expressed as mean ± S.E.M; (n = 5).

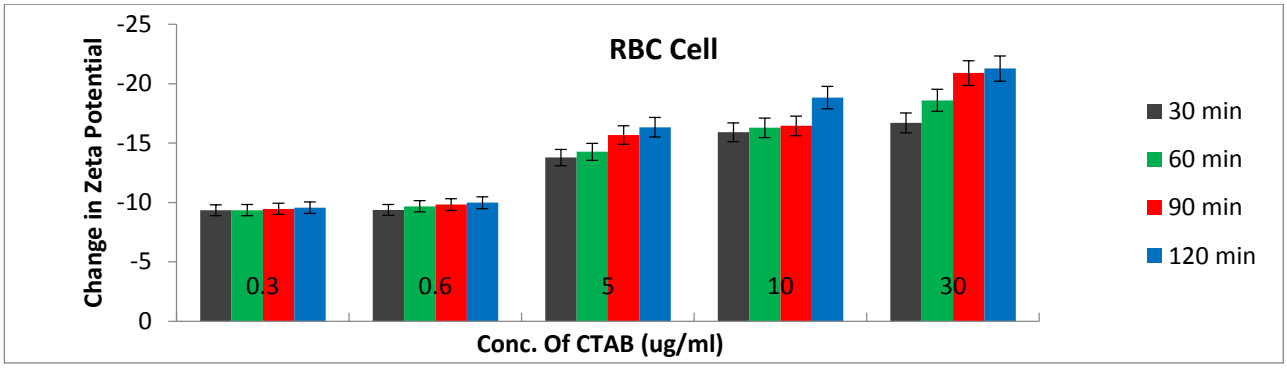
1.2:Zeta potential measurement:

Table 4b: Effect of CTAB on the Zeta potential of the RBC

ZP of RBC(- 9.35)

Conc. Of CTAB (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	-9.34±0.46	-9.35±0.4	-9.46±0.4	-9.56±0.49
0.6	-9.37±0.46	-9.68±0.4	-9.82±0.4	-9.98±0.49
5	-13.78±0.6	-14.27±0.71	-15.67±0.7	-16.34±0.7
10	-15.92±0.79	-16.29±0.8	-16.45±0.86	-18.84±0.89
30	-16.7±0.83	-18.6±0.87	-20.9±0.98	-21.287±1.06

INFERENCE- CTAB causes RBC zeta potential changes



Values are expressed as mean ± S.E.M; (n = 5).

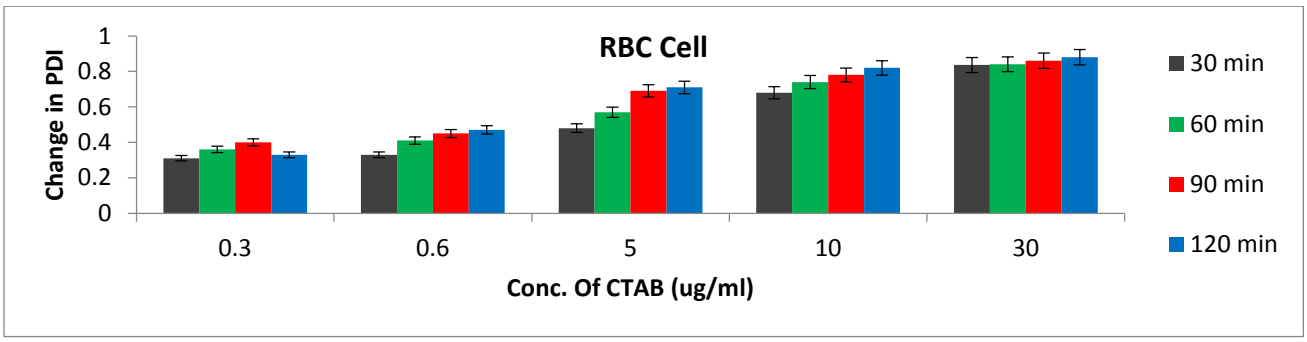
1.3:Poly dispersity index

Table 4c: Effect of CTAB on the PDI of the RBC.

PDI of RBC 0.3

Conc. Of CTAB (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	0.31±0.015	0.36±0.018	0.4±0.02	0.33±0.016
0.6	0.33±0.016	0.41±0.02	0.45±0.02	0.47±0.02
5	0.48±0.02	0.57±0.02	0.69±0.03	0.71±0.03
10	0.68±0.03	0.74±0.03	0.78±0.03	0.82±0.04
30	0.836±0.04	0.84±0.04	0.86±0.04	0.88±0.04

INFERENCE- CTAB causes RBC PDI changes



Values are expressed as mean ± S.E.M; (n = 5).

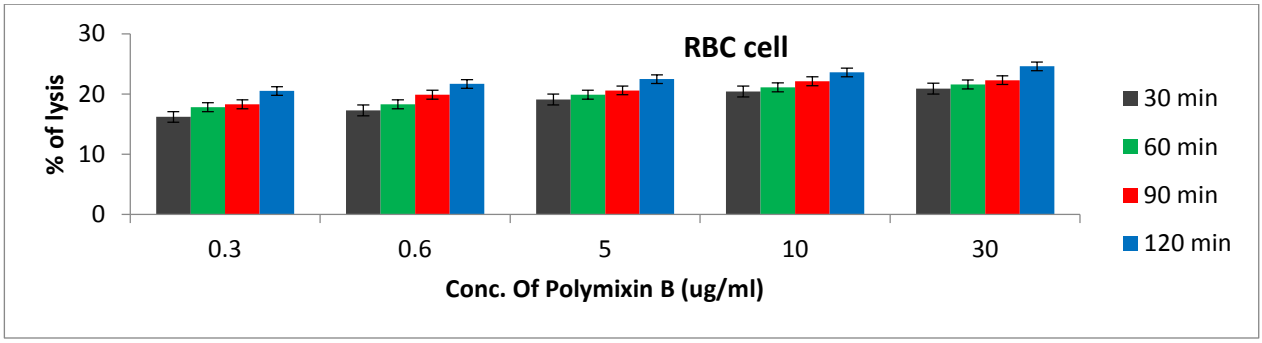
2. Effect of Polymixin B on RBC

2.1:Effect of polymixin B on RBC lysis:

Table 4e: EFFECT OF POLYMIXIN B ON THE RBC LYSIS (calculated as % of lysis)

Conc. Of Poly B (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	16.2 ± 1.2	17.8 ± 1.4	18.3 ±1.1	20.5 ± 2.3
0.6	17.3 ±0.7	18.3± 1.1	19.9±0.9	21.67± 1.8
5	19.1 ±0.7	19.9± 0.8	20.6±1.8	22.47±1
10	20.4 ±1.1	21.1± 1.5	22.1±1.4	23.6±0.8
30	20.9 ±1.6	21.6± 1.7	22.3±1.2	24.6 ± 1.3

INFERENCE- POLYMIXIN B causes no RBC lysis



Values are expressed as mean ± S.E.M; (n = 5)

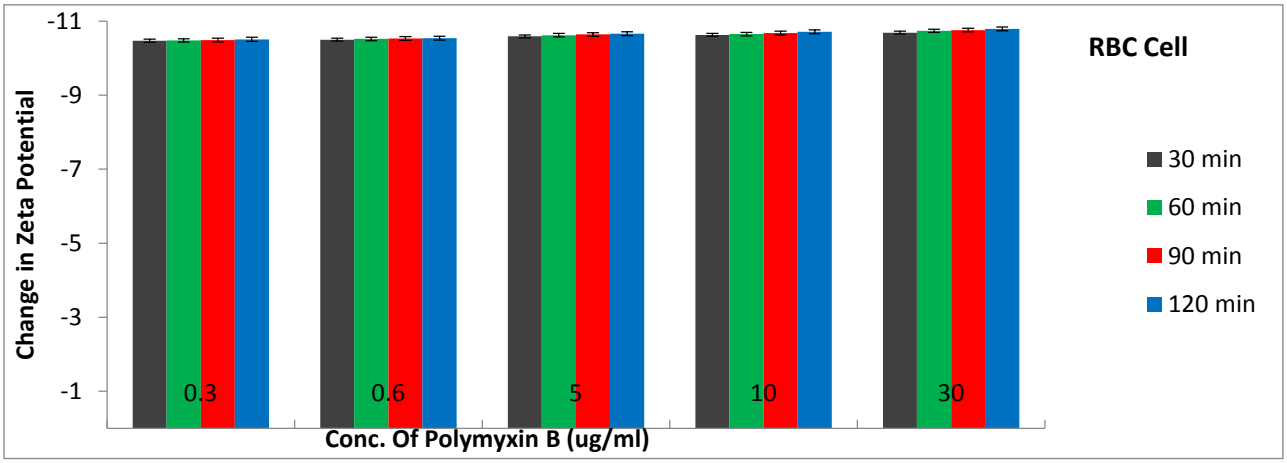
2.2: zeta potential measurement

Table4f: Effect of Polymyxin B on the ZETA POTENTIAL of the RBC

ZP of RBC (- 9.8)

Conc. Of Polymixin B (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	-10.47±0.5	-10.48±0.5	-10.49±0.5	-10.51±0.5
0.6	-10.5±0.5	-10.52±0.5	-10.53±0.5	-10.54±0.5
5	-10.59±0.5	-10.62±0.5	-10.64±0.5	-10.66±0.5
10	-10.63±0.5	-10.65±0.5	-10.68±0.5	-10.71±0.5
30	-10.69±0.5	-10.74±0.5	-10.76±0.5	-10.79±0.5

INFERENCE- Polymyxin B causes no changes of zeta potential of RBC



Values are expressed as mean ± S.E.M; (n = 5).

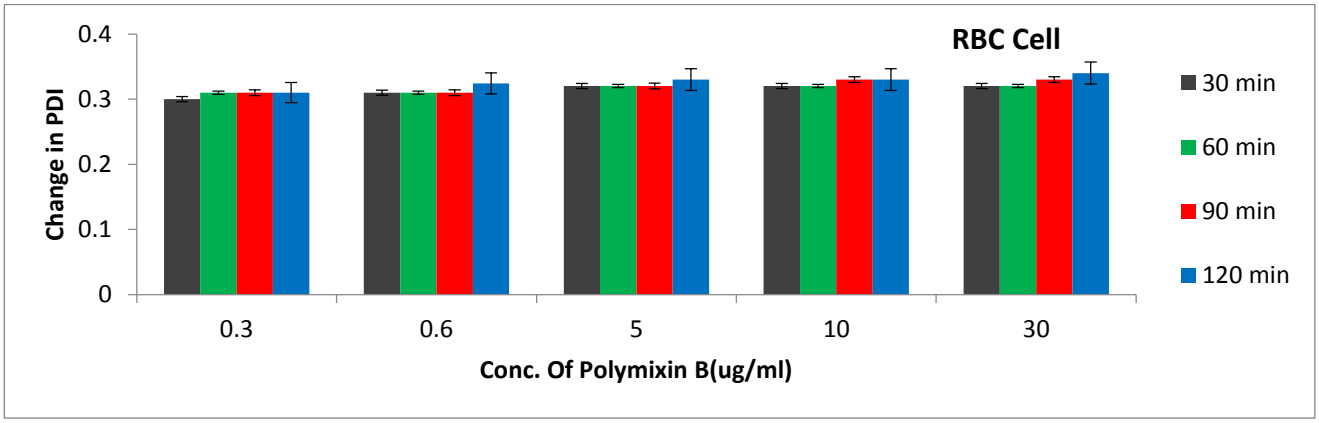
2.3:Polydispersity index measurement:

Table 4g: Effect of Polymyxin B on the PDI of the RBC

PDI of RBC (0.32)

Conc. Of Polymixin B (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	0.3±0.01	0.31±0.01	0.31±0.01	0.31±0.01
0.6	0.31±0.01	0.31±0.01	0.31±0.01	0.324±0.01
5	0.32±0.01	0.32±0.01	0.32±0.01	0.33±0.01
10	0.32±0.01	0.32±0.01	0.33±0.01	0.33±0.01
30	0.32±0.01	0.32±0.01	0.33±0.01	0.34±0.01

INFERENCE- Polymyxin B causes no changes of PDI of RBC



Values are expressed as mean ± S.E.M; (n = 5).

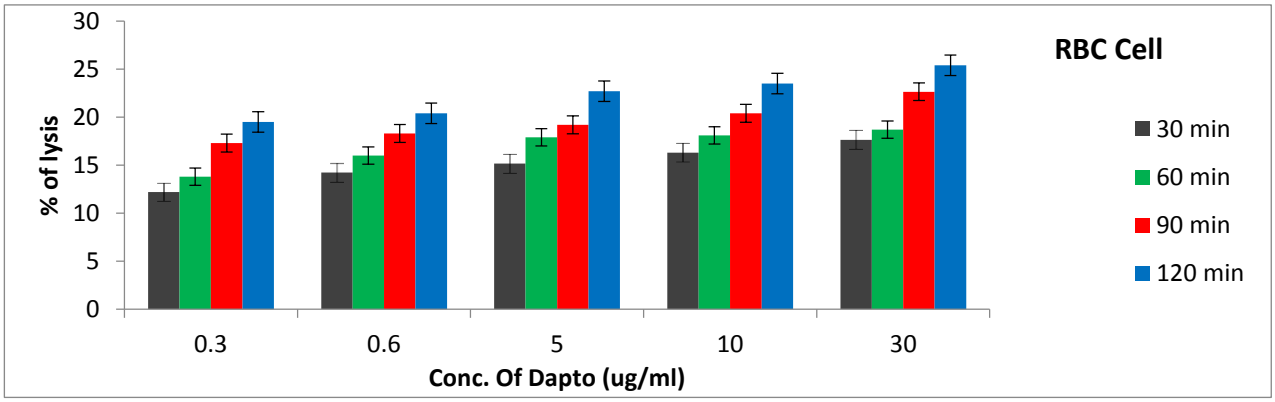
3. Effect of DAPTOMYCIN on the RBC:

3.1Effect of DAPTOMYCIN on the RBC lysis:

Table 4h: Effect of DAPTOMYCIN on the RBC lysis (calculated as % of lysis)

Conc. Of Daptomycin (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	16.2 ± 1.2	17.8 ± 1.4	18.3 ±1.1	20.5 ± 2.3
0.6	17.3 ±0.7	18.3± 1.1	19.9±0.9	21.67± 1.8
5	19.1 ±0.7	19.9± 0.8	20.6±1.8	22.47±1
10	20.4 ±1.1	21.1± 1.5	22.1±1.4	23.6±0.8
30	20.9 ±1.6	21.6± 1.7	22.3±1.2	24.6 ± 1.3

INFERENCE- Daptomycin causes no RBC lysis



Values are expressed as mean ± S.E.M; (n = 5).

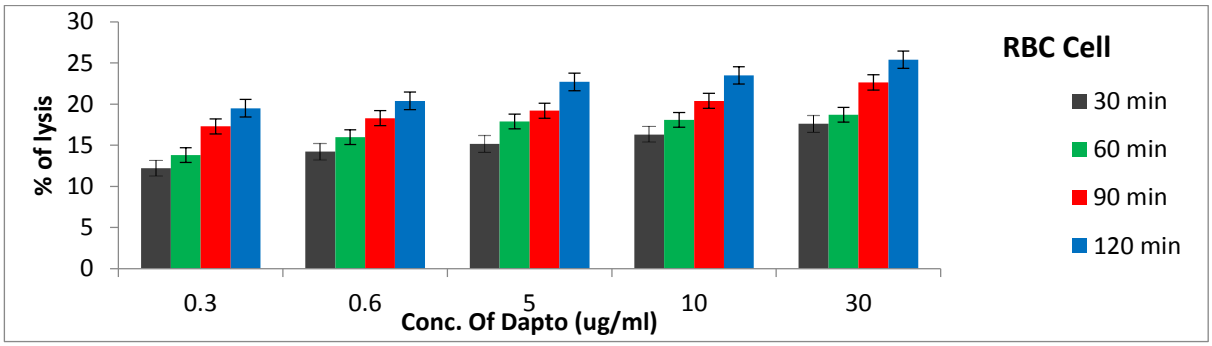
3.2**:zeta potential measurement**

Table 4I: Effect of Daptomycin on the Zeta potential of the RBC

ZP of RBC (- 9.8)

Conc. Of Daptomycin(µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	-9.83±0.4	-9.92±0.4	-9.94±0.4	-9.97±0.4
0.6	-9.9±0.4	-9.94±0.4	-9.95±0.4	-9.96±0.4
5	-9.93±0.4	-9.96±0.4	-9.97±0.4	-9.99±±0.49
10	-9.96±0.4	-9.97±0.4	-9.99±0.4	-10.06±0.5
30	-9.98±0.4	-9.99±0.4	-10.04±0.4	-10.08±0.5

INFERENCE- Daptomycin causes no changes of Zp of RB



Values are expressed as mean ± S.E.M; (n = 5).

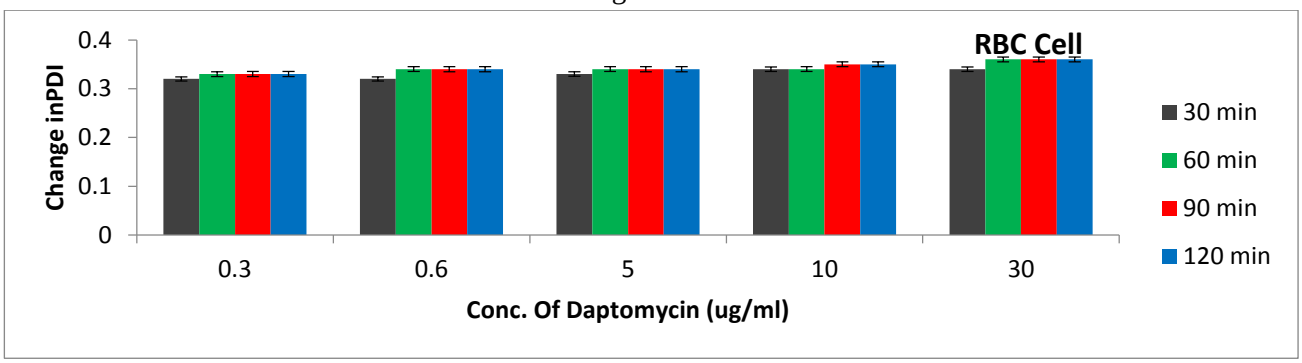
3.3: Polydispersity index measurement:

Table 4j: Effect of Daptomycin on the PDI of the RBC|

PDI of RBC (0.32)

Conc. Of Daptomycin (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	0.32±0.01	0.33±0.01	0.33±0.01	0.33±0.01
0.6	0.32±0.01	0.34±0.01	0.34±0.01	0.34±0.01
5	0.33**±0.01**	0.34±0.01	0.34±0.01	0.34±0.01
10	0.34±0.01	0.34±0.01	0.35±0.01	0.35±0.01
30	0.34±0.01	0.36±0.01	0.36±0.01	0.36±0.01

INFERENCE- DAPTOMYCIN causes no changes of PDI of RBC



Values are expressed as mean ± S.E.M; (n = 5).

III. Effect of CTAB on GSH pretreated S.aureus and E.coli

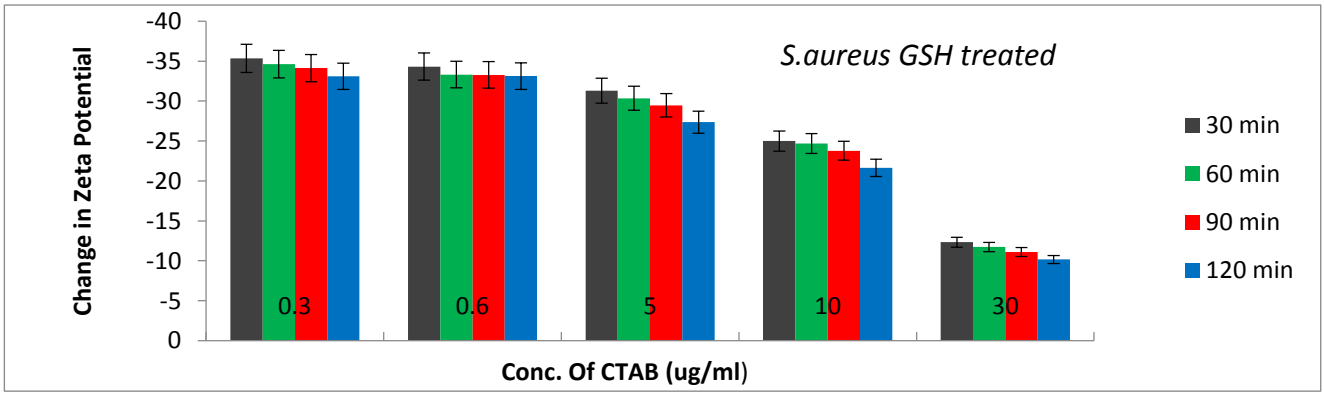
1.zeta potential measurement

Table 5a:Effect of CTAB on the ZETA POTENTIAL of the GSH treated (5 µg/ml) *S.aureus*

ZP of S .aureus (-35.6)

	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	-35.24±1.7	-34.63±1.7	-34.13±1.7	-33.1±1.6
0.6	-34.22±1.6	-33.29±1.6	-33.15±1.6	-33.03±1.7
5	-31.29±1.5	-30.35±1.4	-29.47±1.4	-27.85±1.3
10	-24.886±1.2	-24.68±1.2	-23.77±1.2	-21.63±1.06
30	-12.28±0.6	-11.52±0.5	-11.1±0.5	-10.05±0.48

INFERENCE- GSH has no protective action on S.aureus



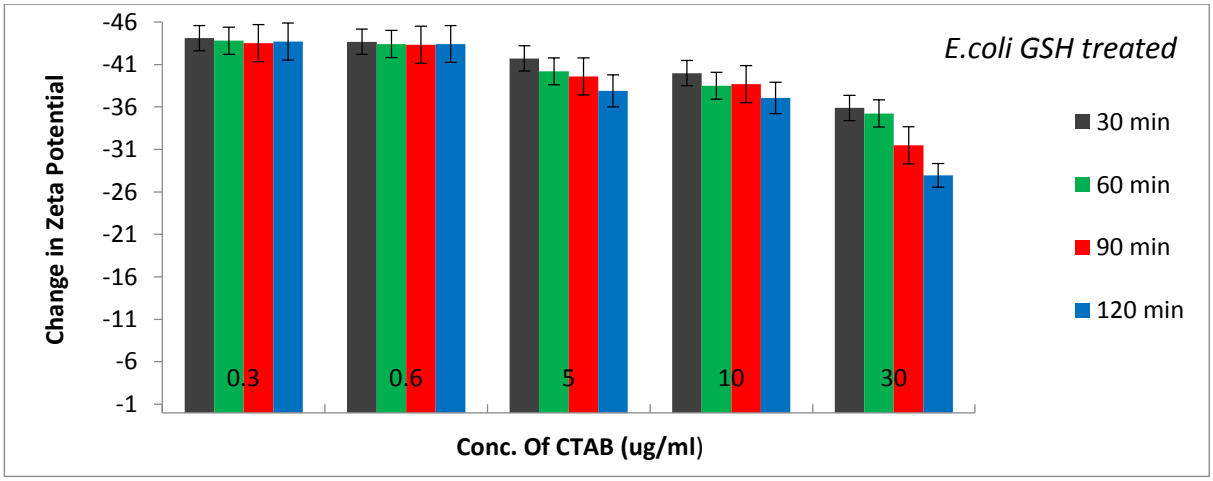
Values are expressed as mean ± S.E.M; (n = 5).

Table 5b: Effect of CTAB on the ZETA POTENTIAL of the GSH treated (5 µg/ml) *E.coli*

ZP of E.coli (-44.2)

Conc. Of CTAB (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	-44.12±2.2	-43.83±2.2	-43.51±2.1	-43.70±2.1
0.6	-43.67±2.1	-43.42±2.1	-43.34±2.1	-43.22±2.1
5	-41.61±2	-40.26±1.87	-39.4±1.92	-37.6±1.9
10	-39.88±1.9	-38.51±1.8	-38.2±1.8	-37.08±1.7
30	-35.7±1.7	-35.26±1.59	-31.58±1.4	-27.56±1.2

INFERENCE- CTAB causes changes in Zp of the E.coli (gram negative) but GSH has no protective action



Values are expressed as mean ± S.E.M; (n = 5).

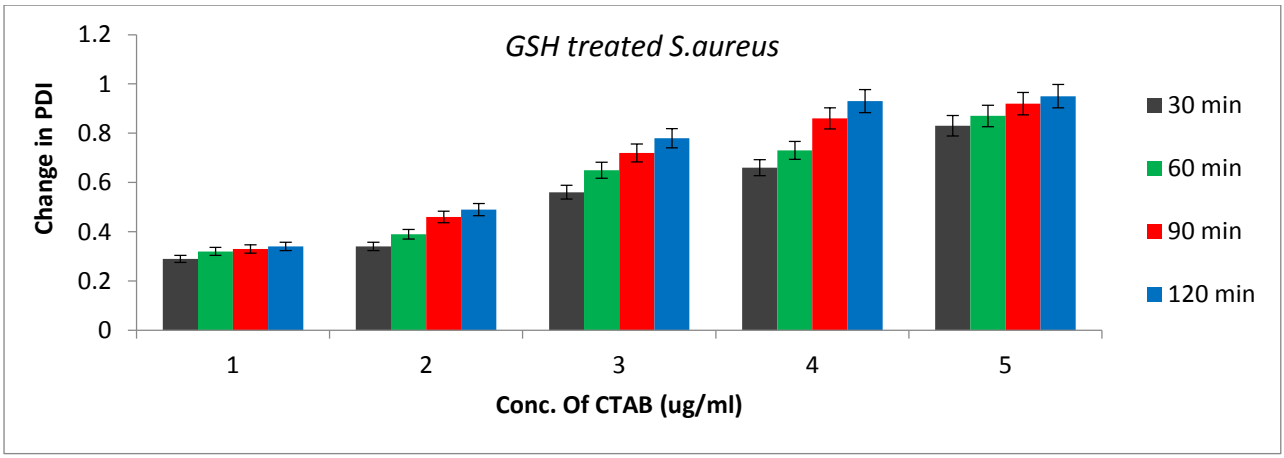
3.2: Polydispersity index measurement

Table 5c:Effect of CTAB on the PDI of the GSH treated (5 µg/ml) *S.aureus* (gram positive)

PDI of S .aureus- (0.28)

Conc. Of CTAB (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	0.29±0.016	0.32±0.016	0.33±0.0165	0.34±0.016
0.6	0.339±0.017	0.40±0.0195	0.45±0.0235	0.49±0.024
5	0.561±0.027	0.65±0.032	0.72±0.036	0.78±0.039
10	0.65±0.0327	0.73±0.036	0.86±0.043	0.93±0.046
30	0.82±0.040	0.87±0.043	0.93±0.046	0.96±0.047

Inference: CTAB cause changes in PDI of the S.aureus, GSH has no protective action



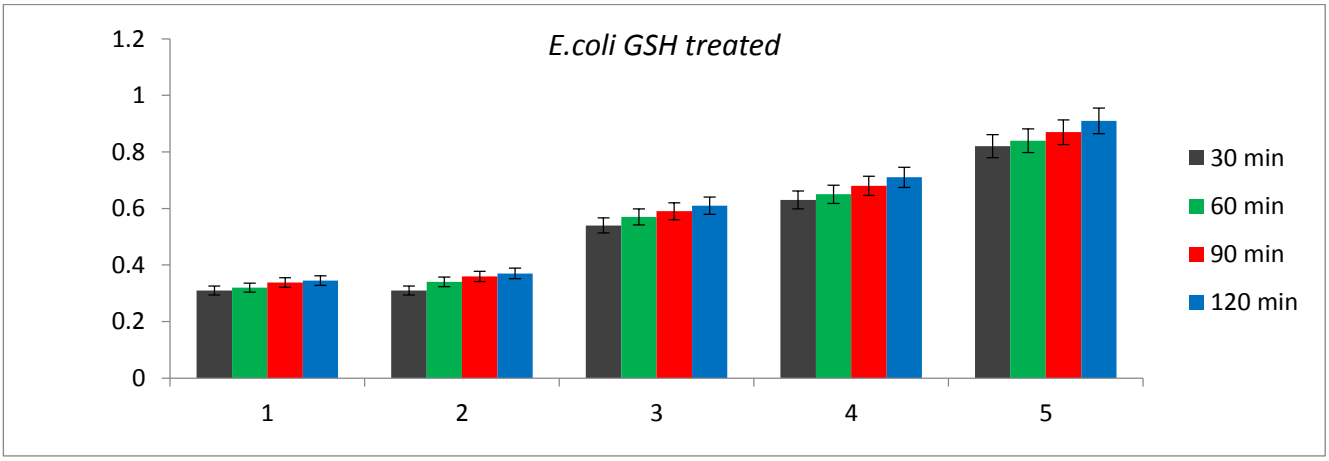
Values are expressed as mean ± S.E.M; (n = 5).

Table 5d:Effect of CTAB on the PDI of the GSH treated(5 µg/ml*) E.coli* (gram negative)

PDI of *E.coli*- (0.31)

Conc. Of CTAB (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	0.314±0.013	0.329±0.016	0.338±0.0165	0.345±0.017
0.6	0.31±0.015	0.34±0.016	0.36±0.018	0.37±0.015
5	0.539±0.027	0.569±0.028	0.59±0.0295	0.61±0.030
10	0.63±0.031	0.652±0.032	0.68±0.038	0.70±0.035
30	0.81±0.041	0.84±0.042	0.87±0.0435	0.90±0.0455

INFERENCE- CTAB cause changes in PDI of THE *E.coli* , GSH has no protective action



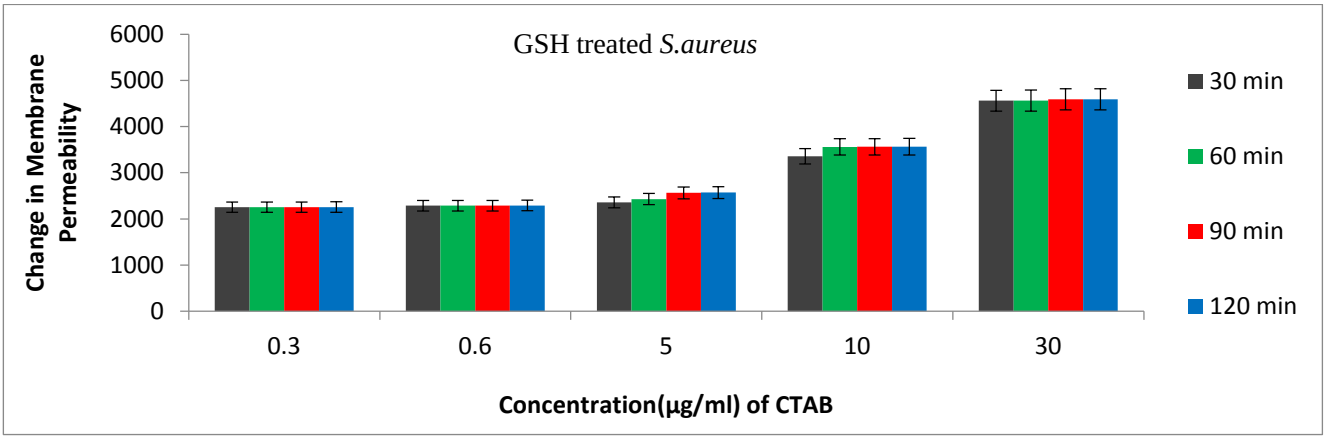
Values are expressed as mean ± S.E.M; (n = 5).

3.3.Memebrane permeability:

Table 5e:Effect of CTAB on the membrane permeability of the *S.aureus*(gram positive)

EFFECT OF CTAB ON THE membrane permeability OF THE S.aureus (gram positive)				
S .aureus (2250.632)				
	Time of exposure			
Conc. Of CTAB (ug/ml)	30 min	60 min	90 min	120 min
0.3	2255.874±112.23	2257.892±112.23	2259.122±112.34	2260.765±112.85
0.6	2301.324±115	2315.868±115.1	2317.439±114.32	2320.991±115.32
5	2481.83±122.43	2592.5±128.34	2601.5±129.32	2735.5±134.5
10	3497.83±172.34	3744.33±184.3	4099.1±204.34	4154.5±206.65
30	4727±235.23	4843.43±239.76	5021.83±248.43	5058.7±248.45
INFERENCE- CTAB cause changes in Permeability of S.aureus				

But GSH has no protective activity.



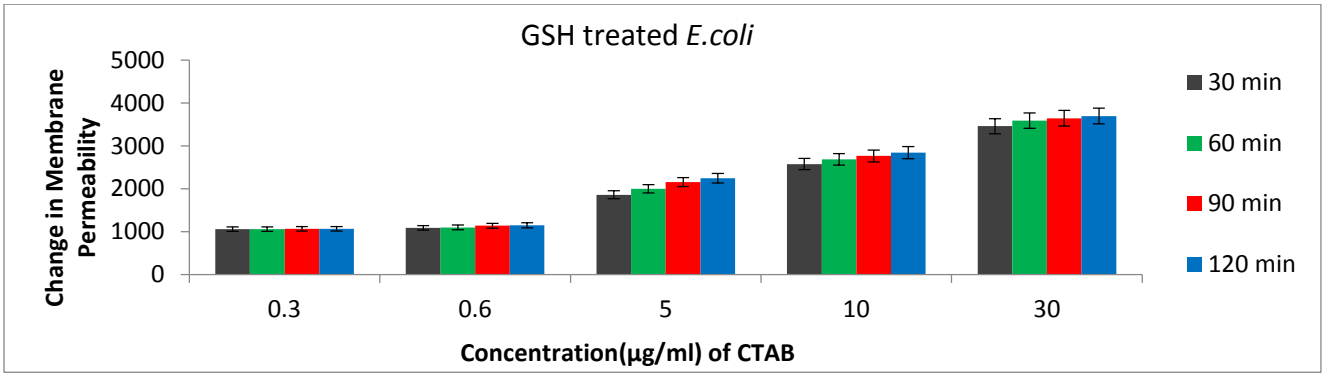
Values are expressed as mean ± S.E.M; (n = 5).

Table 5f:Effect of GSH treated (5 µg/ml) CTAB on the membrane permeability of the *E.coli* (gram negative)

E.coli-(1058.7)

Conc. Of CTAB (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	1059.5± 52.564	1061.37±53.05	1063.16±53.15	1067.65±53.2
0.6	1089.5±53.92	1097.61±54.5	1138.67±56.34	1159.65±57.96
5	1890.3±91.178	2039.8±101.88	2245.97±112.25	2419.1±120.94
10	2602.5±129.8	2736.17±136.78	2956.78±147.8	3052.8±152.5
30	3508.8±174.6	3679.2±183.34	3795.72±189.75	3926.2±196.3

INFERENCE- CTAB causes changes in Permeability of the E.coli (gram negative) but GSH has no protective activity.



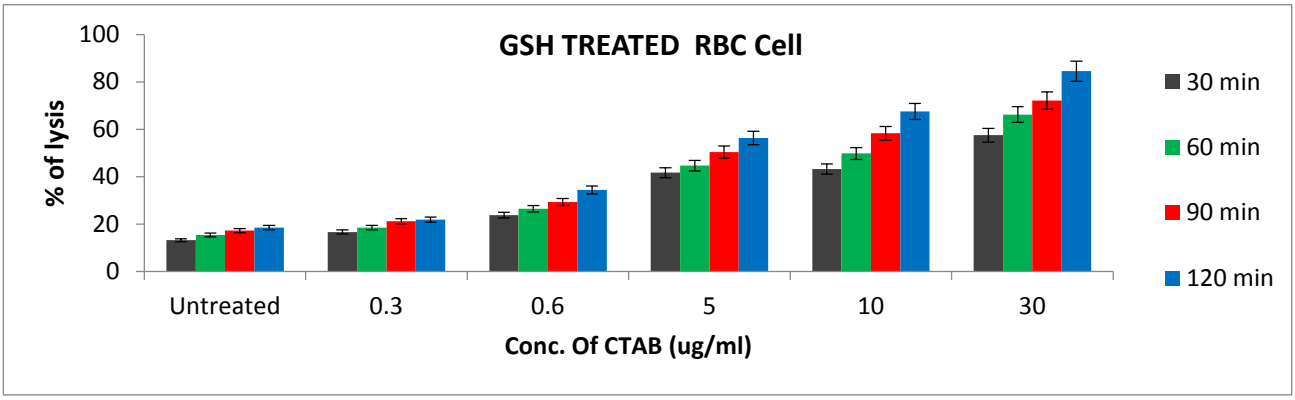
Values are expressed as mean ± S.E.M; (n = 5).

IV.Effect of CTAB on Erthrocytes (GSH treated)

Table 6a: Effect **of CTAB on the GSH treated RBC LYSIS (calculated as % of lysis)**

Conc. Of CTAB (mcg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0	13.2 ± 1.8	15.4 ± 1.7	17.3 ±2.3	18.5 ± 1.8
0.3	16.7 ± 1.6	18.5 ± 2.8	21.2 ± 1.7	21.9 ± 2.2
0.6	23.8 ± 2.5	26.5 ± 1.1	29.3 ± 3.6	34.4 ± 1.5
5	41.7 ± 1.4	44.7 ± 1.8	50.4 ± 2.1	56.3 ± 2.2
10	43.2 ± 1.3	49.8 ± 1.9	58.3 ± 2.2	67.5 ± 1.8
30	57.5 ± 2.1	66.2 ± 2.7	72.1± 1.5	84.5 ± 2.5

INFERENCE- GSH has protective action on RBC



Values are expressed as mean ± S.E.M; (n = 5).

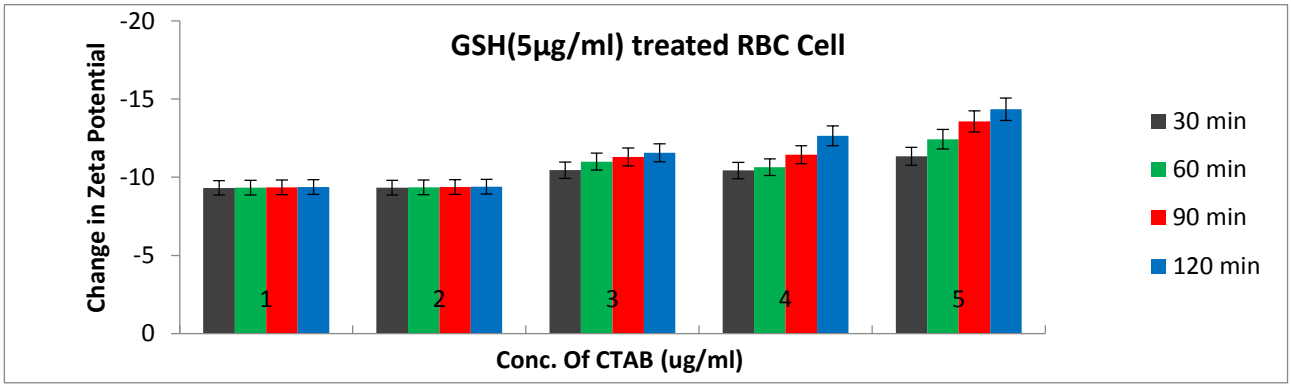
Table 6b: Effect of CTAB on the Zeta potential of the GSH treated (5 µg/ml) RBC

ZP of RBC

(-9.35)

Conc. Of CTAB (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	-9.32±0.4	-9.33±0.4	-9.35±0.4	-9.37±0.4
0.6	-9.34±0.4	-9.35±0.4	-9.37±0.4	-9.39±0.4
5	-10.45±0.5	-10.50±0.55	-11.3±0.5	-11.56±0.5
10	-10.48±0.5	-10.64±0.5	-11.44±0.6	-12.65±0.6
30	-11.34±0.6	-12.43±0.6	-13.57±0.7	-14.34±0.74

INFERENCE- GSH has protective action on RBC



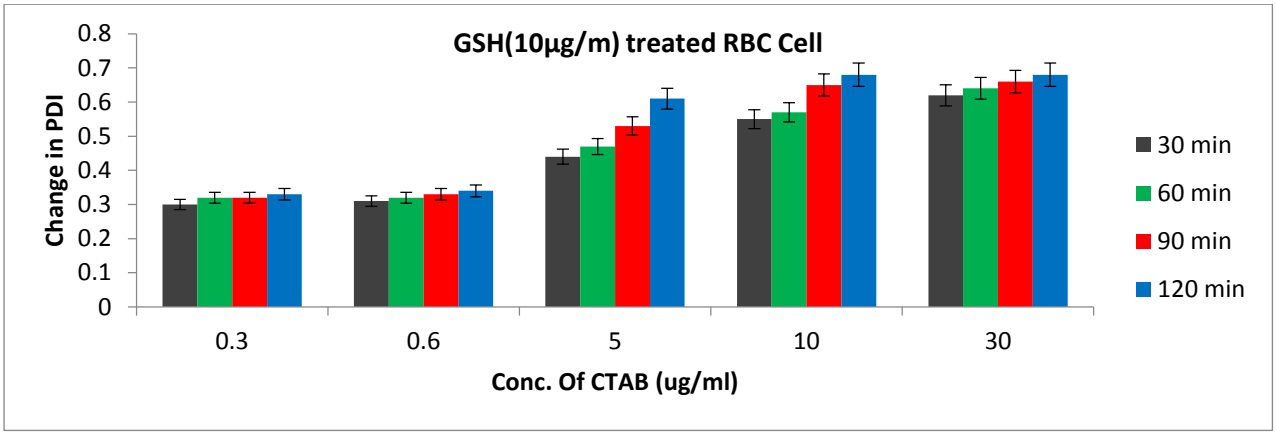
Values are expressed as mean ± S.E.M; (n = 5).

Table 6c: Effect of CTAB on the PDI of the GSH treated (10ug/ml) RBC

PDI of RBC 0.32

Conc. Of CTAB (µg/ml)	Time of exposure			
	30 min	60 min	90 min	120 min
0.3	0.3±0.01	0.32±0.01	0.32±0.01	0.33±0.01
0.6	0.31±0.01	0.32±0.01	0.33±0.01	0.34±0.01
5	0.44±0.02	0.47±0.02	0.53±0.02	0.61±0.03
10	0.55±0.02	0.57±0.02	0.65±0.03	0.68±0.03
30	0.62±0.03	0.64±0.03	0.66±0.03	0.68±0.03

INFERENCE- GSH has protective action on RBC



Values are expressed as mean ± S.E.M; (n = 5).

Estimation of size, intensity & conc. of RBC suspension:

Effect of CTAB on RBC suspension was analyzed using Nanoparticle Tracking Analysis (NTA). Normal RBC was observed at 695 nm, but when the RBC samples was treated with CTAB that peak was not observed and peak with small size was observed (195nm), which indicates the lysis and fragmentation of RBC. 3D Plot of particle distribution and scattering plot also indicate the same.

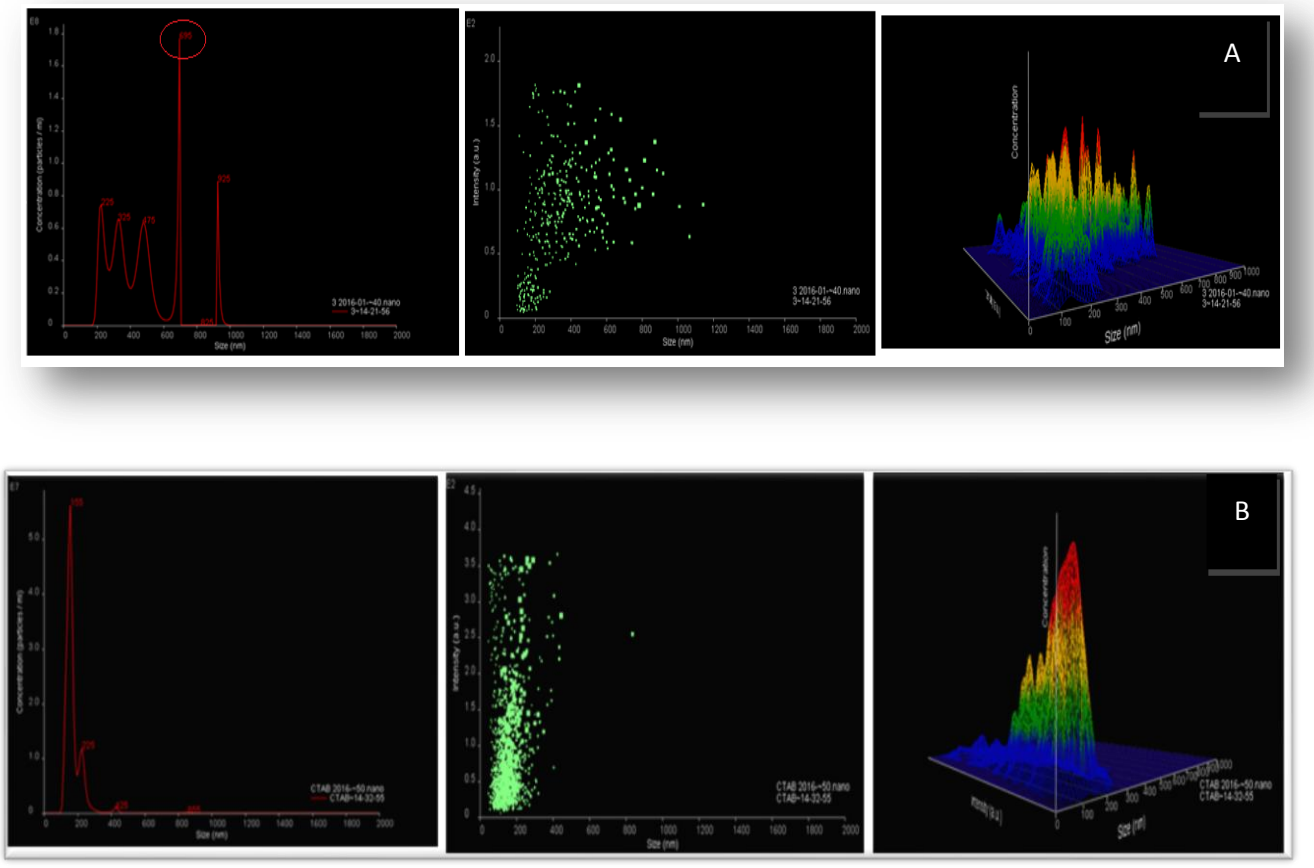


Figure: Size distribution from NTA measurements of RBC suspension (left panels) with the scattering (middle panels) and 3D graph (size vs. intensity vs. concentration; right panels). **A) Blank RBCo ; B) RBC treated with CTAB.**

Microscopic analysis

Ghost cell

A dead cell in which the outline remains visible, but whose nucleus andcytoplasmic structure s are not stainable. Red blood cell after loss of its hemoglobin is also known as ghost cell.

Biconcavity

Red blood cells or Erythrocytes are biconcave and they do not contain nucleus that means they are a-nucleated. This biconcave structure provides the cell to attain large surface area to accommodate maximum space for hemoglobin. The biconcavity also helps the RBC in maintaining osmotic robustness as a spherical RBC is more prone to rupture under an osmotic stress normally experienced inside a red cell.

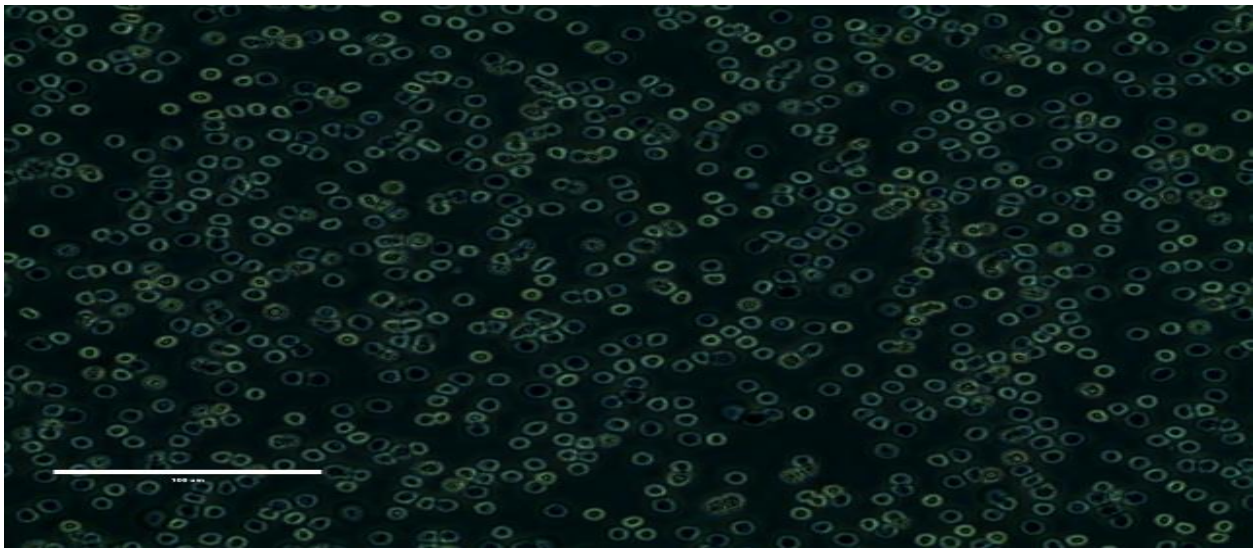
Anisocytosis

It is a physical condition in which the red blood cells of a person are found to be of unequal size. This normally happens when the patient suffers from anemia, Thalassemia or any other disorder of blood cells.

Poikilocytocis

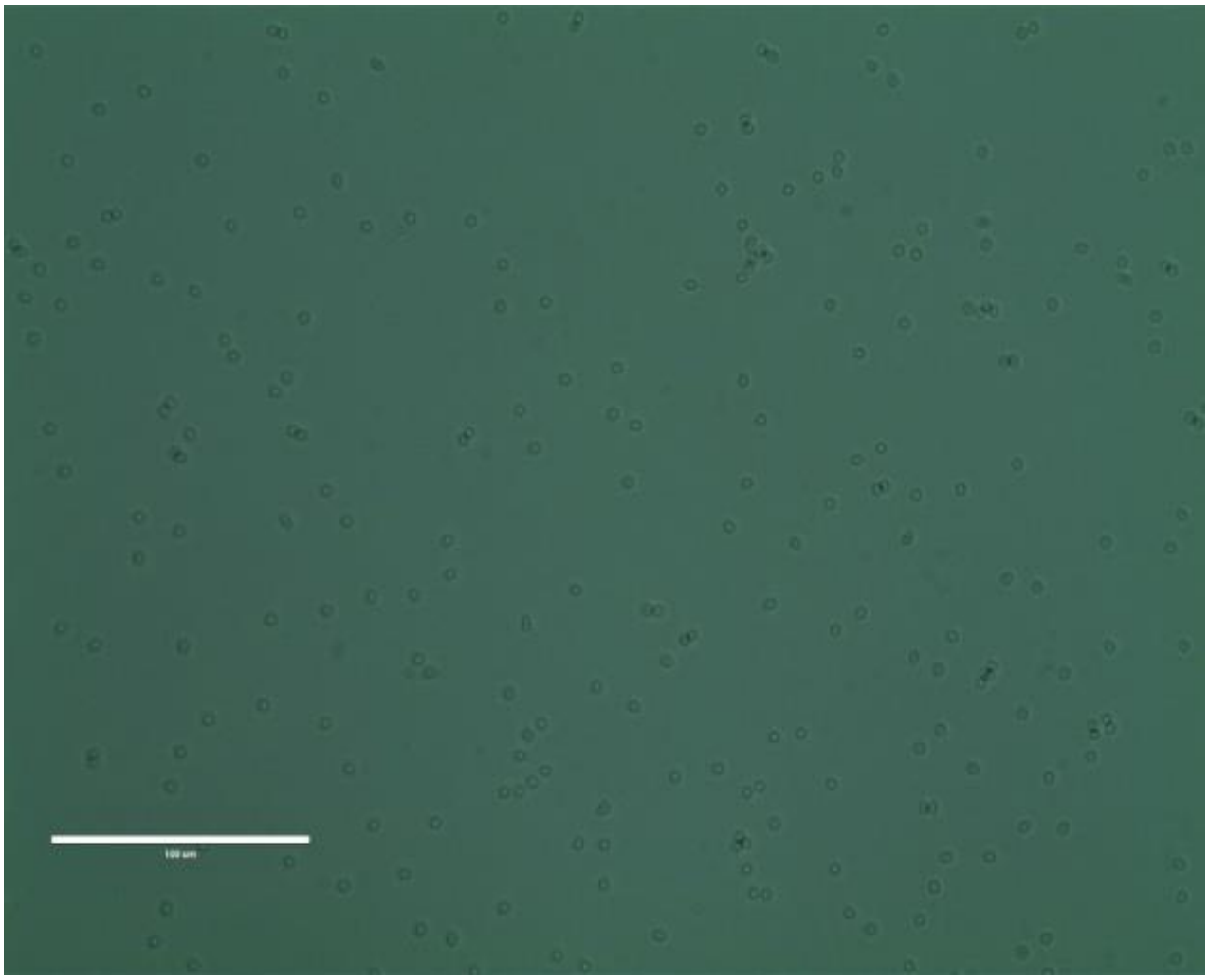
Poikilocytosis is a blood disorder in which 10% or more red blood cells (RBCs) are abnormally shaped. These abnormal shaped RBCs are termed as poikilocytes. There are many different abnormal shapes (burr, sickle, tear drop, elliptical).

Fig.1. RBC blank



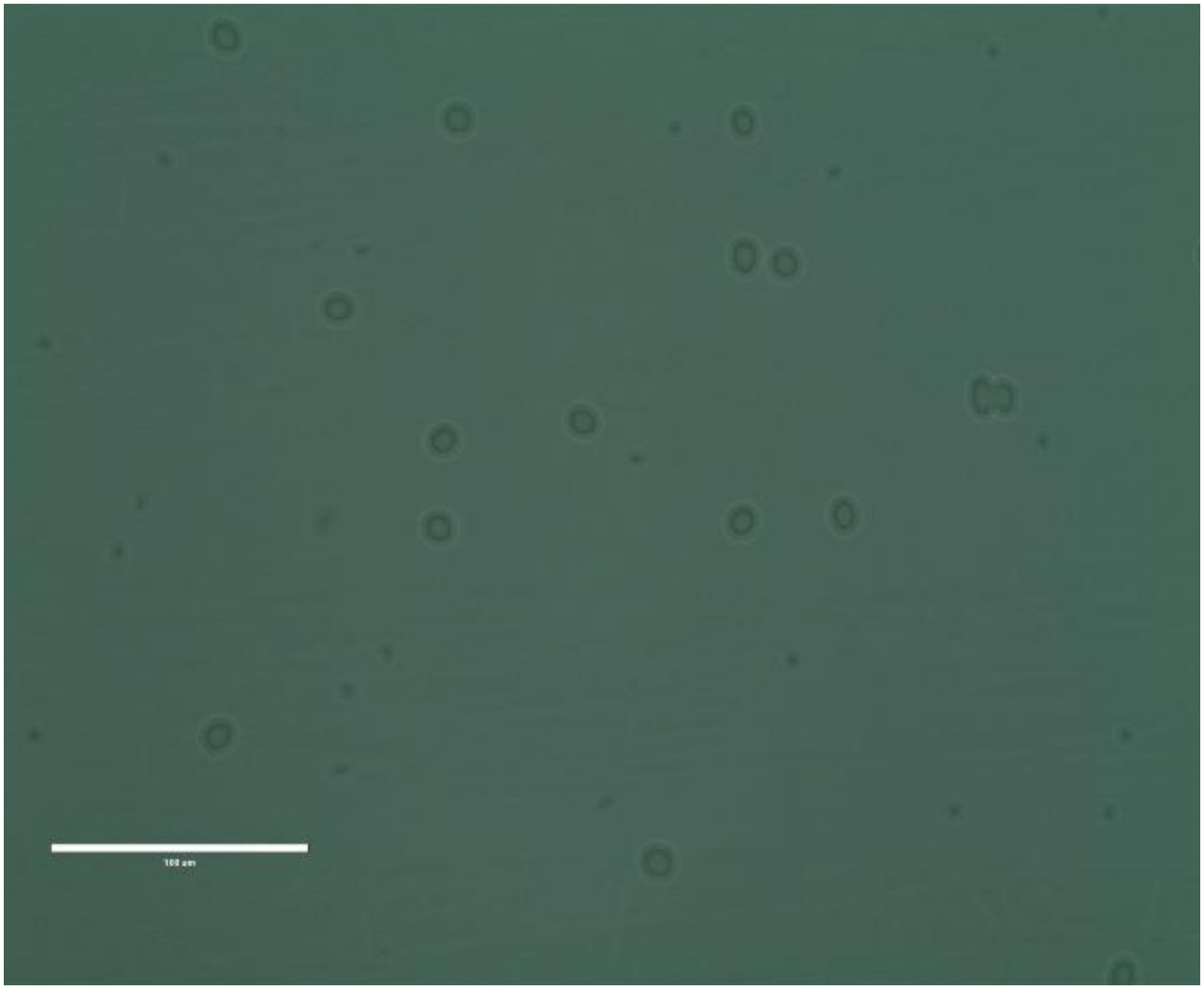
In the normal RBC cell the uniform shape and distribution are maintained. No ghost cell has been seen. Anisocytosis as well as Poikilocytocis both absent. Biconcavity present.

Fig.2. RBC treated with CTAB (concentration 30µg/ml)



In the CTAB treated RBC; the uniform shape and distribution of RBC are not maintained as like untreated RBC. No ghost cell has been seen but the apparent appearance of RBC cells indicates that the cell might have ruptured .Distribution is uniform. Biconcavity maintained.

3. **RBC treated with Daptomycin (30 mcg/ml)**



In the Daptomycin treated RBC; though the uniform shape(round) of RBC is maintained, distribution of RBC are not identical as like untreated cells. No ghost cell has been seen but the apparent appearance of RBC cells indicates that the cell are not ruptured.

CHAPTER 5

DISCUSSION

In the modern era of drug discovery and development, targeting the bacterial cell surface is of great concern due to the escalating evidence of multiple drug resistance pathogens and a gradually decreasing availability of potent antibiotic to counter such bacterial infection. Targeting the bacterial membrane seems to acquire more importance because membrane targeting agents exhibit a rapid bactericidal effect and have less possibility to acquire resistance, moreover as their action is destructive hence viability of bacteria is often reduced (Van, 2008; Waxman, 1980). Thus, making the membrane targeting agents a suitable alternative for therapeutic exploitation.

Previous researches have suggested that the interaction of various membrane acting agents with bacterial surface may involve variety of mechanism such as the interaction of functional groups of the agents with bacterial surface followed by aggregation within the membrane may result in the disruption of bacterial surface, perturbation of membrane integrity (Vinod et al., 2013), leading to the formation of pores on the surface (Giuliani, 2008; Alves, 2010). Such membrane perturbation often enhances cell permeability leading to cell death. (Powers and Hancock, 2003).

Cationic amphipathic peptides, such as cecropins, induce cell death in prokaryotic and eukaryotic cells by increasing membrane permeability (C.H. Packman. 2008). Increased permeability may lead to cell lysis or, alternatively, may produce subtle changes in the membrane's barrier function that promote cell death. The *in vitro* cytotoxic and lytic activity of short cationic peptides (from mammalian source) and insect-derived alpha-helical peptides have also been studied with the objective of establishing the anticancer potential of these agents (Rice, L.B., 1996Bonomo).According to literature, the lytic activity of the peptides has been founded to be 2-50 times more to tumour cells than to the non-malignant cells. Further, the cytotoxic activity of these peptides was found to more or less equivalent when tested against sensitive and multidrug-resistant cell lines. In addition to their inherent cytotoxic activity of these membrane-active peptides can also enhance the *in vitro* cytotoxic activity of doxorubicin against multidrug-resistant tumour cells. (Zasloff M, Martin B, Chen HC)

In the present investigation, an effort has been made to establish the correlation between changes in Zeta potential (ZP), poly dispersity index (PDI) and cell surface permeability using bacteria (*Escherichia coli, Pseudomonas aeruginosa* (gram

negative bacteria) and *Staphylococcus aureus, Bacillus subtilis* (gram positive bacteria) with that of RBC cell suspension.

According to Abraham and Bhat (2008), the concentration of detergent plays an important role in determining whether the cell is being permeabilised or lysed during the treatment with detergent. An enhanced uptake of NPN thought to occur with bacterial cells with damaged (functionally invalid) outer membrane. (Loh et al., 1984). In the present investigational work, apart from the bacterial cells, the erythrocytes were also exposed to different concentration of Polymixin B, Daptomycin and CTAB and the alteration in membrane permeability along with changes in zeta potential with subsequent changes in polydispersity index of were investigated.

Red blood cell (RBC) membranes contain lipids rich in unsaturated fatty acids. RBCs are more frequently exposed to oxygen than other body tissue and, thus, are more susceptible to oxidative damage (Guilani and Rinaldi AC: 2008). Invasion of the RBC membrane by pro-oxidants may lead to cell hemolysis. Moreover, the hemoglobin present in RBCs is also considered to be strong catalyst which may initiate lipid peroxidation. Erythrocytes are found to be the common targets of such oxidative damage because they are rich in high concentrations of unsaturated lipids as well as iron (in the hemoglobin). Since these cells cannot replace the damaged cellular components and hence, oxidative damage may induce a permanent alteration in the red cell membranes (Sen et al. 1995). In addition, erythrocyte membrane proteins are susceptible to covalent damage including cross-linking and aggregation by oxygen radical-induced lipid peroxidation (Davis and Goldberg. 1987).

In the present study, CTAB when studied on *S. aureus*, at a concentration of 0.3 mcg/ml, displayed a Zeta potential of -35.34 and -33.1 (30 min and 120 min), whereas when the same compound was used at a concentration of 30mcg/ml, it altered the zeta potential (ZP) to -12.32 and -10.15 when measured at the 30 min and 120 min respectively. Similarly for *E. coli* ZP was found to be -44.1 and -43.7(0.3 µg/ml; 30 min and 120 min) and -35.9 to -27.9 (30µg/ml; for 30 minutes and 2 hour incubation). Therefore from our observation the exposure of gram positive and gram negative cell to CTAB resulted in the alteration of ZP and similar observation also recorded for *B. subtilis* and *P. aeruginosa*.

In case of membrane permeability CTAB treated bacterial cell showed higher uptake of NPN in *S.aureus* (using same concentration of drug that had been used for ZP analysis) and it was found to be 2255.8 to 2260.7(0.3 µg/ml; 30 minutes and 2 hour incubation) and 4727 to 5058.7 (30 µg/ml; 30 minutes and 2 hour incubation). When studies on *E.coli*, it was found to be 1059.5 and 1067.65 (0.3 mcg/ml; 30 min and 120

min) and 3508.8 to 3926.2 for 30 minutes and 2 hours respectively. *B.subtilis* and *P.aeruginosa* exposed to CTAB, also showed similar type of changes for similar time intervals. Here also changes of ZP could be observed, at the doses similar to the ones used for studying to the effect of CTAB on *S. aureus* and *E.coli*. Therefore from the present observation changes in the NPN uptake (due to altered membrane permeability) was found to coincide with the alteration of zeta potential.

The haemolytic activity of membrane-active AMPs against erythrocytes is often used as a measure for their cytotoxicity and to estimate their therapeutic index. Comparing the percentage haemolysis for each AMP, minimal haemolytic properties (less than 2% haemolysis) against sheep erythrocytes than human intestinal epithelial cells. This may be related to different plasma membrane compositions of normal and transformed mammalian cells [Vaara M Vaara T, 1981] metabolic activity, as well as the particular assay used for comparison.

In order to evaluate the cytotoxic effect of CTAB, Daptomycin and Polymixin B (membrane destabilizing agents), the compounds were also evaluated against sheep erythrocytes. In a manner similar to the study performed on bacterial cells, here we also recorded the zeta potential (ZP), poly dispersity index (PDI) and the percentage of haemolysis of sheep erythrocytes exposed to different concentration of the compounds at different time intervals.

Red blood cell suspension was observed by treating with CTAB, Daptomycin and Polymixin B and from our observation, CTAB produced haemolytic changes whereas Daptomycin and Polymixin B did not display any haemolytic properties. At similar concentrations, CTAB produced insignificant alterations of ZP [-9.34 and -9.56(0.3µg/ml)] however a remarkable alteration of ZP has seen [-16.7 and -21.28 (30 µg/ml)], at 30 minutes and 2 hours. However, no such alteration of ZP could be observed in sheep RBC for Polymixin B (0.3µg/ml,-10.47 at 30 minute; 30µg/ml, -10.79 at 2 hours) and for Daptomycin (0.3µm/ml,-9.83 at 30 minute; 30 µm/ml, -10.08 at 2 hours**) .** Similarly, CTAB produced alteration of PDI in sheep erythrocytes, at 30 minutes (0.3µm/ml, 0.31) and also at 2 hours (0.3µg/ml, 0.33), whereas (30µg/ml, 0.83) at 30 minutes and (30µg/ml, 0.88) at 2 hours. However, no such changes could be observed with either Daptomycin or Polymixin B.

Effect of GSH on the hemolytic property of antibacterial compounds

GSH is is a tripeptide with a gamma peptide linkage between the carboxyl group of the glutamate side-chain and with the amine group of cysteine is converted to its oxidized form glutathione disulfide (GSSG) [2]. [3] The ratio of reduced glutathione to oxidized glutathione within cells is often used as a measure of cellular toxicity. GSH has been suggested to be a more effective scavenger of free radicals than any other endogenous antioxidant in an aqueous environment (Mukhopadhyay et al. 1995). GSH is an anti-oxidant which has been found to protect the cells from the damaging effect of free radicals.

Since GSH was found to present a protective effect on erythrocytes (exposed to CTAB), the same model was also utilized to assess the effect of GSH on gram positive and gram negative bacteria. GSH pretreatment (5 µg/ml) did not produce any protective effect on either *S.aureus* or *E.coli*, when studied with CTAB, at time intervals of 30, 60, 90 and 120 minutes respectively. The alteration of zeta potential and PDI were found to increase with time as well as with increased concentrations of CTAB (0.3, 0.6, 5, 10, 30 µg/ml).

Therefore, unlike sheep erythrocytes, GSH was not found to produce any protective effect on CTAB induced bacterial membrane destabilization .Furthermore, our earlier observations were also supported from the results obtained from assessment of membrane permeability, studied using NPN. GSH was unable to alter CTAB induced membrane permeability in both gram positive (*S.aureus* and *B.subtilis*) and gram negative (*E.coli* and *P.aeruginosa*) cells.

In earlier studies, antioxidants like GSH augmented the activity of β-lactam antibiotics (penicillin and ampicillin). However, no such effects could be observed with ascorbic acid. Moreover, the study also indicated that potentiating effect of GSH was independent of gamma glut amyl trans peptidase (GGT) activity (GGT is responsible for GSH uptake from extracellular medium and is also involved in transpeptidation during cellular hemolysis). Rather, GSH is involved in altering the expression of cys B (a protein regulator for GSH transport), which may also be responsible for increasing the susceptibility of the cells to the β lactam antibiotics (Goswami and Jawali, JAC, 2007) .In some studies increased concentrations of GSH (≥50) produced a haemolytic effect in a concentration dependent manner and such activity of GSH was attributed to increase acidic environment (Scarier et al, 2013). Hence, the altered activity of GSH in prokaryotic system may be mediated through mechanisms unrelated to oxidative stress (Goswami and Jawali, JAC, 2007).

In the present context, assessment of zeta potential , poly dispersity index (PDI) ,cell permeability along with particle size distribution, particle movement, microscopic analysis were performed. Based on our results, it can be suggested that alteration of ZP may be correlated to the enhancement of membrane permeability and PDI and such change in ZP and PDI coincided with cell death (both gram positive and gram negative bacteria) and also the lytic effect of CTAB on RBC cell suspension.

Therefore based on the present findings, it may be mentioned that the cationic detergents like Cetyl trimethyl ammonium bromide (CTAB) produces membrane destruction in both eukaryotic and prokaryotic systems, as was evident from the different experimental models. Moreover, such damaging effect of CTAB could be reduced with the addition of GSH in eukaryotic system, however the antioxidant failed to produce any such protective effect in prokaryotic cells. Further studies are presently in progress to understand the mechanism of such contradicting effects of GSH on prokaryotic and eukaryotic cells.

CHAPTER-6

Reference

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