IDENTIFICATION OF FUNCTIONAL MOLECULES AND PHARMACOLOGICAL ACTIVITIES INVESTIGATION OF THE METHANOLIC EXTRACT OF *EGG SHELL*

A thesis report submitted to the department of Pharmacy, East West University,Bangladesh, in partial fulfillment of the requirements for the degreeof M. Pharm in Clinical Pharmacy and Molecular Pharmacology

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This is to certify that the dissertation, entitled **"Identification Of Functional Molecules And Pharmacological Activities Investigation Of The Methanolic Extract Of *Egg Shell*."** Is a thesis work done by Kaniz Sultana (ID:2013-3-79-034) in partial fulfillment of the requirements for the degree of Masters of Pharmacy. We further certify that all the sources of information and laboratory facilities availed in this connection is duly acknowledged.

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This is to certify that the dissertation, **"Identification Of Functional Molecules And Pharmacological Activities Investigation Of The Methanolic Extract Of *Egg Shell*",** is a thesis work done by Kaniz Sultana (ID:2013-3-79-034) in partial fulfillment of the requirements for the degree of Masters of Pharmacy. We further certify that all the sources of information and laboratory facilities availed in this connection is duly acknowledged.

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Declaration by the Candidate

I, Kaniz Sultana (ID:2013-3-79-034), hereby declare that the dissertation entitled **"Identification Of Functional Molecules And Pharmacological Activities Investigation Of The Methanolic Extract Of *Egg Shell*"**, submitted by me to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the degree of Masters of Pharmacy is a genuine & authentic thesis work carried out by me during Spring 2014- Fall 2014 under the supervision and guidance of Dr. Repon Kumer Saha, Assistant Professor, Department of Pharmacy, East West University.

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ACKNOWLEDGEMENT

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RATIONALE AND OBJECTIVE OF THE WORK

It was discovered that exposure to egg shell membranes (ESM) significantly reduced the thermal resistance and/or inhibited selected Gram-positive and Gram-negative foodborne bacterial pathogens. Methods to extract these enzyme-rich shell membranes are readily available and offer egg processors potential economic value as a value-added product and provide processors with a 'natural' antibacterial adjuvant to sensitize bacterial pathogens and spoilage organisms to other stresses for food or pharmaceutical applications. However, a greater understanding of the egg shell membrane components that deliver antimicrobial activity is critical with respect to developing a better understanding of how it may be used in practical applications. Numerous attempts to purify active fractions of β-Nacetylglucosaminidase (β-NAGase), lysozyme and ovotransferrin from the ESM proved somewhat limited; however, isoelectric focusing and ion exchange chromatography provided a technical means whereby relatively pure protein/enzyme samples were obtained.

The aim of this research project was to carry out the characterization of the functional molecules present in the methanolic extract of seed of the *Egg Shell* and investigate their biological activities.

ABSTRACT

. The objective of this study is to characterize the functional compounds that were extracted and separated from Egg Shell and were carried out using different methods using Thin Layer Chromatography (TLC). So, In vitro anti-diabetic activity of mathanolic extract of different fraction were measured.The result showed, Solid fraction of of methanolic extract of the *Egg Shell* showed highest and Liquid fraction o of methanolic extract of the Egg Shell showed lowest anti-diabetic activity 51.5 % and 6 % respectively.

.The methanolic extract showed some antioxidant, antidiabetic and stornger antibacterial activity. In antibacterial result shows that it has great effect on pathogenic bacteria. It is called natural antibacterial effect. The result showed that it has grat effect against *Shigella dysentriae, E.coli.*

INTRODUCTION

1.1 HISTORY:

In 1999 (Mead and colleagues ,1999), in conjunction with the Centers for Disease Control and Prevention, presented their data on the incidences of foodborne illnesses in the United States. They estimated that over 76 million cases of foodborne illness occur annually resulting in over 325,000 hospitalizations and over 5,000 deaths. These staggering figures come from a country that boasts itself as a leading producer of the world's safest food. Even more alarming is that some consider these numbers to be an underestimation.

Although more than 250 foodborne diseases have been described, known bacteria (e.g., *Campylobacter, Salmonella, E. coli* O157:H7), viruses (e.g., Norwalk-like, caliciviruses), and parasites (e.g., *Giardia, Cyclospora*) account for an estimated 14 million illnesses. It is difficult to calculate the exact cost as a result of such illnesses; however, medical costs and lost wages due to foodborne salmonellosis, only one of many foodborne infections, have been estimated to be more than $1 billion per year (CDC,2003).

Implementing preventative means such as Good Manufacturing Practices (GMPs), Good Agricultural Practices (GAPs), and Hazard Analysis Critical Control Points (HACCP) have certainly made a positive impact at reducing the risks of foodborne illnesses. In 2002, a report from the CDC's Emerging Infections Program Foodborne Diseases Active Surveillance Network (FoodNet) showed a decrease in the major bacterial foodborne illnesses. However, some infections failed to show a steady decline in their occurrence, indicating that increased efforts are needed to further reduce the incidence of foodborne illnesses, especially considering the emergence of new, more resilient pathogens (e.g., multidrug-resistant *Salmonella*). This information, along with demands and concerns from consumers, has opened the door for food and pharmaceutical producers to explore new options in food and drug safety.

In the last decade, consumers have shown an increasing demand for minimally processed foods and sustained functionality of naturally occurring bioactive ingredients. Several factors have fueled the public's interest in exploring options for minimally processed foods and bioactive compounds to enhance nutrition including concerns about the safety and tolerance of synthetic preservatives; the suspected link between the overuse of subtherapeutic antibiotics as animal growth promotants and the development of multi-drug resistance in microbes, as well the increased media attention given to diet and health (Naidu, 2000).

A number of products have been approved by regulatory agencies for use as direct food antimicrobials. However, the limited spectrum of antimicrobial activity of some of these substances has led to the continued search for more effective antimicrobials among naturally occurring compounds (Davidson and Zivanovic, 2003). Although not often associated with food safety, great potential lies in the area of egg shell waste.

In 2001,(Poland and Sheldon,2001) discovered that exposure to egg shell membranes significantly reduced the thermal resistance and/or inhibited selected Gram-positive and Gram-negative foodborne bacterial pathogens [i.e., up to 3 log reduction in *L.monocytogenes* populations and 83 - 87% reduction in thermal decimal reduction times(D-values) for *Salmonella enterica* serovar Typhimurium, *Salmonella enterica* serovar Enteritidis, and *Escherichia coli* O157:H7; Poland and Sheldon, 2001]. Methods to easily extract these enzyme-rich shell membranes are readily available (Winn and Ball,1996; MacNeil, 1998, 2001) and offer egg processors potential economic value as a value-added product and provide processors a 'natural' processing adjuvant to sensitize bacterial pathogens and spoilage organisms for food or pharmaceutical applications.

However, a greater understanding of the egg shell membrane (i.e. the components responsible for the antimicrobial activity and their activity and stability) is essential to better understand how it may be use.

1.2 EGG SHELL:

An **eggshell** is the outer covering of a hard-shelled egg and of some forms of eggs with soft outer coats. Bird eggshells contain calcium carbonate and dissolve in various acids, including the vinegar used in cooking. While dissolving, the calcium carbonate in an egg shell reacts with the acid to form carbon dioxide.

FIGURE 1: Chicken brown egg shell

1.3 The Anatomy of a Chicken Egg
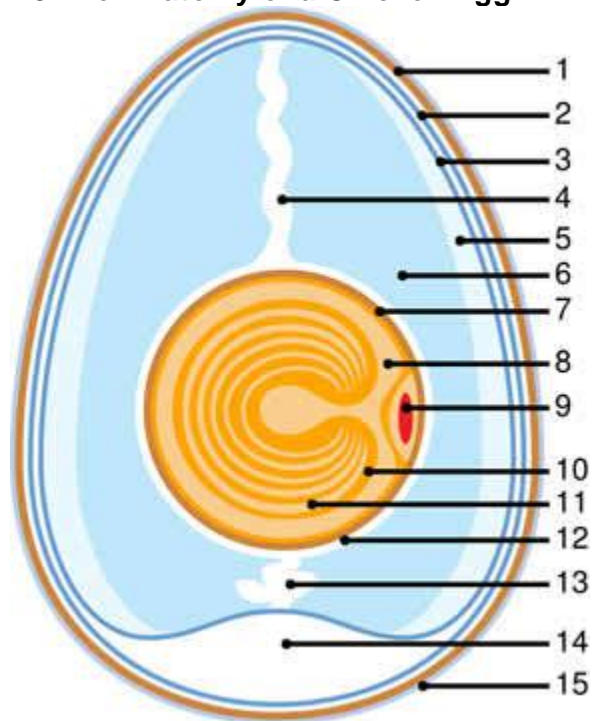
1. **Eggshell** – The outer eggshell is made almost entirely of calcium carbonate (CaCO3) and is covered with as many as 17,000 tiny pores. It is a semipermeable membrane, which allows air and moisture to pass through its pores. The shell also has a thin outermost coating called the bloom or cuticle that helps keep out bacteria and dust (see below 15).

2. **Outer shell membrane**

3. **Inner shell membrane** – These two membranes – outer and inner – are just inside the shell surrounding the albumen (white). The two membranes provide an efficient defense against bacterial invasion and are made partly of keratin. The outer membrane sticks to the egg shell while the inner membrane sticks to the albumen. When an egg is first laid, it is warm. As it cools, the contents contract and the inner shell membrane separates from the outer shell membrane to form the air cell (see 14 below).

4. **Chalaza** – are twisted in opposite directions and serve to keep the yolk centered. The more prominent the chalazae, the fresher the egg.

5. **Exterior albumen** (outer thin albumen) — The outer thin albumen is a narrow fluid layer next to the shell membrane.

6. **Middle albumen** (inner thick albumen) -The inner thick white (chalaziferous layer) is a dense, matted, fibrous capsule of albumen around the vitelline membrane of the yolk.

The matted fibrous capsule terminates on each end in the chalazae, which are twisted in opposite directions and serve to keep the yolk centered. This part of the egg is a excellent source of riboflavin and protein. In high-quality eggs, the inner thick albumen stands higher and spreads less than thin white. In low-quality eggs, it appears thin white.

7. **Vitelline membrane** – The clear casing that encloses the egg yolk. When an egg is said to be "mottled", the yolk surface is covered with many pale spots or blotches. The strength and integrity of the vitelline membrane are very important in preventing egg yolk mottling.

8. **Nucleus of pander** – a plug of whitish yolk, with no particular significance for development and whose function is purely a nutritive one, like the rest of the yolk. (See: Int. Schmitt S., (2005) J. Dev. Biol. 49: 1-8).

9. **Germinal disk** (blastoderm) – a small, circular, white spot (2-3 mm across) on the surface of the yolk; it is where the sperm enters the egg. The nucleus of the egg is in the blastodisc. The embryo develops from this disk, and gradually sends blood vessels into the yolk to use it for nutrition as the embryo develops.

10. **Yellow yolk** – a major source of vitamins, minerals, almost half of the protein, and all of the fat and cholesterol. The yolk contains less water and more protein than the white, some fat, and most of the vitamins and minerals of the egg. These include iron, vitamin A, vitamin D, phosphorus, calcium, thiamine, and riboflavin. The yolk is also a source of lecithin, an effective emulsifier. Yolk color ranges from just a hint of yellow to a magnificent deep orange, according to the feed and breed of the hen.

11. **White yolk** – Also known as, the latebra is an area of white yolk located in the center of the yolk. It is lower in fat and therefore stands out as a bright white area in many Magnetic Resonance Images. The specific function of the latebra is uncertain but it may act as a central structure around which the additional layers of the yolk are formed.

12. **Internal albumen** (Chalaziferous albumen)– The inner thick white (chalaziferous layer) is a dense, matted, fibrous capsule of albumen around the vitelline membrane of the yolk. The matted fibrous capsule terminates on each end in the chalazae, which are twisted in opposite directions and serve to keep the yolk centered.

13. **Chalaza chalazae**, which are twisted in opposite directions and serve to keep the yolk centered. The more prominent the chalazae, the fresher the egg. chalazae, which are twisted in opposite directions and serve to keep the yolk centered.

14. **Air cell** – An air space forms when the contents of the egg cool and contract after the egg is laid. The air cell usually rests between the outer and inner membranes at the

15.

eggs larger end. As the egg ages, moisture and carbon dioxide leave through the pores of the shell, air enters to replace them and the air cell becomes larger.

16. **Cuticle or bloom** – The shell is produced by the shell gland (uterus) of the oviduct, and has an outer coating, the bloom or cuticle. The cuticle somewhat seals the pores and is useful in reducing moisture losses and in preventing bacterial penetration of the egg shell. Most of cuticle is removed from table eggs when they are mechanically washed

1.4 DIFFERENT USES OF EGG SHELL:

Eggshells/shell membranes have multiple uses as:
- A fertilizer supplying calcium and an amendment for acidic soils.
- A remedy for osteoporosis and joint motility ailments.
- A soil stabilizer for use as construction materials.
- A calcium supplement for hybrid layers in the late production phase that increases egg production and improves shell quality.
- In artwork for production of mosaics and as glue in musical instruments.
- In cosmetics and burn surgery where it improves skin appearance (plastic surgery).
- In orthopedics and dentistry as a treatment aid.
- In the treatment of cancer patients to boost musclegain and hair thickening
- In sports nutrition to increase performance on athletes
- In food industry as a food flavour.
- In photography for production of photography aids.

1.5 CHEMICAL DEFENSE OF DIFFERENT TYPES OF EGG SHELL:

The intact egg is naturally equipped with several physical and chemical defenses aimed at protecting the developing embryo from physical harm and microbial invasion.

Primary physical barriers to microbial penetration of the egg include the cuticle, shell and the inner and outer shell membranes. The light-pink colored membranes, composed of highly cross-linked proteins similar to keratin, collagen and elastin, are structurally similar to a meshwork of entangled threads which aid in obstructing invading microorganisms (Baker and Balch, 1962).

Apart from the physical barriers, the avian egg is also equipped with a number of chemical defenses which inhibit or prevent microbial growth in the albumen. Egg albumen (composed of approximately 88% water, 10% protein, 1% carbohydrate, 0.5%lipid and 0.5% minerals) contains lysozyme, avidin, conalbumin (ovotransferrin),ovomucoid and other components that possess varying degrees of antimicrobial activity.

The alkalinity of the albumen, which increases from an initial pH of approximately 7.6 to greater than 9.0 after one week at 25°C, is a major naturally-occurring antimicrobial

factor (Board and Tranter, 1994).

Ovomucoid inhibits proteolytic enzymes (i.e., trypsin inhibitor) important for microbial growth (Garibaldi, 1960). Lysozyme is a bacteriolytic enzyme which catalyzes the hydrolysis of β-1,4-

glycosidic bonds of polysaccharides, thereby compromising the peptidoglycan layer of bacterial cell walls and polymers of *N*-acetyl-D-glucosamine (Jolles and Jolles, 1984). Avidin is believed to inhibit microbial growth by binding biotin required by most all bacteria as an essential enzyme cofactor. Conalbumin (or ovotransferrin) acts as a chelator, binding iron and possibly other minerals into a stable complex and potentially producing a deficiency of essential minerals required for invading microorganisms. Initially, it was believed that the antimicrobial properties of ovotransferrin were solely due to iron deprivation, iron being an essential growth factor
for most bacteria (Alderton *et al.,* 1946). However, several more recent reports have described the retention of ovotransferrin antimicrobial activity, even when the protein is complexed with metal ions such as zinc and iron (Valenti et al., 1987; Valenti et al.,1985; Ibrahim, 1996).

The hen's egg also contains a β-N-acetylglucosaminidase classified as a lysosomal enzyme that contributes to the hydrolytic degradation of glycoproteins, mucopolysaccharides and glycolipids (Weissmann *et al.,* 1964; Lush and Conchie, 1966;Robinson and Stirling, 1968; Winn and Ball, 1975). N-acetylglucosaminidase is one offour different genera involved in cleaving chemical bonds inside the peptidoglycan layer(Schockman and Höltje, 1994). Thus, the complex interactions among all of these physical and chemical components within the intact egg provides the greatest protection to the developing embryo, although Lifshitz *et al.* (1963) proposed that the inner shell membrane may be the single most important barrier to bacterial penetration into the egg, with shell membranes acting as bacterial filters, and containing active antibacterial substances.

Histochemical studies using eggshell membranes confirmed the presence of protein and firmly-bound sugars (Simkiss, 1958; Robinson and King, 1968). Starcherand King (1980) identified desmosine and isodesmosine as the major cross-links within these membranes, a finding later recognized as providing the eggshell membrane, itself,with a high degree of insolubility and flexibility, an attribute that supports the egg white before the formation of the shell (Takahashi et al., 1996). In addition, allysine (α-aminoapipic-δ-semialdehyde), the reactive precursor of cross-links, and its aldol condensation product, are present in significant quantities within shell membranes(Crombie et al., 1981). Harris *et al.* (1980) showed that lysyl oxidase, located in a copper-rich region of the isthmus of the hen oviduct, is required for the biosynthesis of these cross-links. These investigators also found lysyl oxidase activity in shell membranes, found to be coupled with catalase. This coupled enzyme complex was considered to be involved in the biosynthesis of eggshell membranes, protecting the embryo against hydrogen peroxide (Harris *et al.,* 1980). Although these components have not been specifically identified as 'antibacterial',

several other proteins identified in the ESM have been documented as exhibiting antimicrobial behavior.

Elliott and Brant (1957) were the first to report the presence of lysozyme in ESM, while Hincke and colleagues (2000) recently determined that lysozyme was heterogeneously distributed throughout the ESM using a colloidal-gold immunocytochemical localization detection method. Winn and Ball (1975) initially identified measurable amounts of β-N-acetylglucosaminidase activity within shell membranes. Ovotransferrin was conclusively localized within the membrane using immunofluorescence (Gautron *et al.*, 2001).

To date, there are a limited number of studies that describe protein purification from eggshell membranes. In the early work of Vadehra *et al.* (1972), lysozyme and other minor protein components were extracted from the ESM using normal saline (0.9%NaCl), phosphate buffers and distilled water solutions. Later, Wong *et al.* (1984) and Takahashi *et al.* (1996) solubilized collagen-like egg shell membrane proteins through a combination of performic acid oxidation and pepsin digestion. Most recently, Yi *et al.*(2004) examined these collagen-like proteins.

A number of products have been approved by regulatory agencies for use as direct food antimicrobials. However, the limited spectrum of antimicrobial activity of some of these substances has led to the continued search for more effective antimicrobials among naturally occurring compounds (Davidson and Zivanovic, 2003). Although not often associated with food safety, great potential lies in the area of egg shell waste. In preliminary studies, Poland and Sheldon (2001) demonstrated that eggshell membranebound components were capable of reducing the heat resistance and/or inhibiting the growth of selected Gram-positive and Gram-negative foodborne bacterial pathogens suspended in 0.1% peptone water. Reductions in thermal decimal reduction times (Dvalues) of 83 - 87% were observed for *Salmonella enterica* serovars Typhimurium and Enteritidis (D54°C) and *Escherichia coli* O157:H7 (D52°C) and up to a 3 log reduction in *L. monocytogenes* populations following incubation with eggshell membranes (ESM) for 30min at 37ºC.

The pink-colored ESM consists of an outer and inner membrane with a thickness of approximately 50 µm and 15 µm respectively. The outer membrane is located just inside the shell and the inner membrane is located between the outer membrane and albumen. Their structure is similar to a meshwork of entangled threads which aids in obstructing invading microorganisms.(Lifshitz *et al.* 1963) concluded that the inner shell membrane may be the most important single barrier to bacterial penetration into the egg, with shell membranes acting as a bacterial filter and also containing antibacterial substances.

1.6 EGG SHELL AND EGG SHELL WASTE:

In our country egg shell consumption is high. As a major contributor to the nation's food supply, 28% of consumed eggs come from processing plants manufacturing products such as used in cakes,,bakery etc. This total amount of egg shell is wasted. Approximately 90% of this waste volume is calcium carbonate, while the other 10% is comprised of proteinaceous eggshell membranes (ESM). It is a serious problem for environment. The protein membrane of egg shell attarct the insects.

The egg shell rich in calcium, eggshells can be used to fortify foods from animal feed to orange juice.When finely ground the shells can also be used as a substitute for pulp in paper, used in the cosmetic industry as an ingredient in facial scrubs, or as a soil amendment by farmers to change soil pH levels on their fields.

1.7 Composition of egg shell:

The shell comprises approximately 9-12% of the total egg weight. It consists primarily of calcium carbonate (94%) with small amounts of magnesium carbonate (1%), calcium phosphate (1%) and organic matter, chiefly protein (4%) (Stadelman, 1973).

The shell not only provides structural integrity to the egg, but contains numerous pores (ca. 7,000 to 17,000 per egg) that permit the diffusion of respiratory gasses. It is important to note that the pores are often many times the diameter of bacterial cells, and even in eggs with undamaged cuticles, as many as 10-20 pores lack either an adequate cover or plug of cuticle which provide the portals for bacteria to infect the internal contents of the egg (North, 1978; Board and Tranter, 1994). The thickness of the eggshell, as influenced by age, hereditary, nutritional and environmental factors, also may play a role in preventing bacterial penetration (Taylor and Martin, 1929).

1.8 Components of the Eggshell Membrane:

The eggshell is lined with two light-pink colored membranes, each of which is composed of highly cross-linked proteins similar to keratin, collagen and elastin (Baker and Balch, 1962). A diagram and scanning electron micrograph of the eggshell and membrane structure is presented in Figure 1 and 2. The eggshell membrane (ESM) consists of an outer and inner membrane with a thickness of approximately 50 μm and 15 μm respectively.

The outer membrane is located just inside the shell and the inner membrane is located between the outer membrane and albumin. The outer shell membrane is attached firmly to the shell by numerous cones on the shell surface extending into the membrane through fibril associations, while the inner membrane lies immediately over the albumen. The membranes are held firmly together, except at the blunt end where they separate to enclose the air space. Their structure is similar to a meshwork of entangled threads which aids in obstructing invading microorganisms. It has been determined that the membranes are largely composed of protein which, because of its high content of cystine and its insolubility, is described as keratin-like (Cooke andBalch, 1970).

Early electron microscopy (Masshoff and Stolpmann et,al 1961) showed that the membrane fibers consisted of a central core having a fine, fibrillar structure surrounded by a fine-granular sheath consisting of protein and mucopolysaccharide. Although fibers from the membranes were similar in structure, fibers from the inner membrane were thinner.
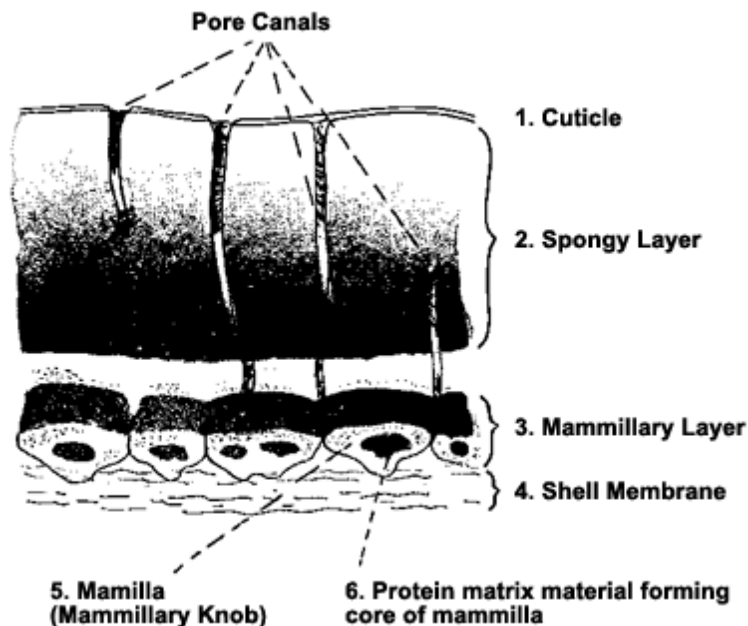
Figure 2. Diagram of the egg shell and egg shell membrane structure.

The mechanism of antimicrobials in food preservation is either through controlling the growth of a contaminating microorganism (bacteriostatic) or killing the microorganism directly (bactericidal) (Tortora *et. al.,* 1986a). To better understand the impact of antimicrobial agents requires an understanding of the structure and composition of the bacterial cell wall.

The bacterial cell wall is a semirigid structure that is responsible for the characteristic shape of the cell. The cell wall surrounds the underlying fragile plasma (cytoplasmic/inner) membrane and protects it, the internal structures, and the cell medium from potentially adverse changes in its surrounding environment. The primary function of the cell wall is to prevent bacterial cells from rupturing when the osmotic pressure inside the cell is greater than that outside the cell (Tortora *et al.,* 1986). The cell wall also serves as a medium for nutrient transport and release of waste products, as well as providing resistance to phagocytes and cellular exchange of DNA (Nikaido and Vaara,1987). Figure 4 and 5 are representations of the components which make up Gram-positive and Gram-negative bacteria cell wall.
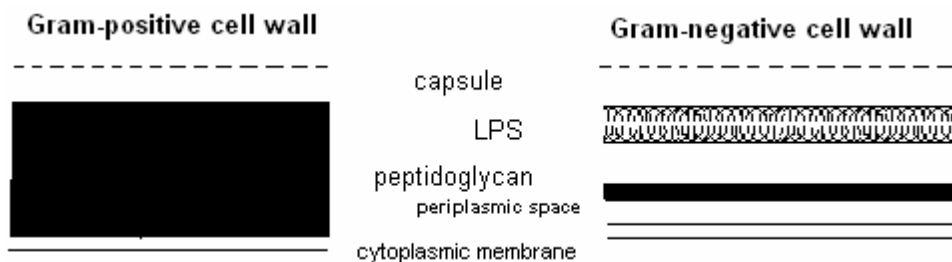
Figure 3: the cell envelope for Gram-positive and Gram-negative bacteria. The Grampositive wall is a uniformly thick layer external to the plasma membrane composed mainly of peptidoglycan (murein). The Gram-negative wall appears thin and multilayered consisting of aphospholipid-lipopolysaccharide (LPS) outermembrane, a relatively thin

peptidoglycan sheet andthe plasma membrane. The space between the inner (plasma) and outer membranes (wherein thepeptidoglycan resides) is called the periplasm.

1.8.1 Peptidoglycan

Peptidoglycan (mucopeptide/murein**)** is a complex macromolecular polymer of amino sugars cross-linked by short peptides. It is the major component of Gram-positive bacterial cell walls and is responsible for cell wall integrity and rigidity. The peptidoglycan polymer is composed of an alternating sequence of two amino sugars related to glucose termed N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAMA). The N-acetylglucosamine and N-acetylmuramic acid molecules are primarily linked together by β-1,4 glycosidic bonds and alternate in rows, each row forming a carbohydrate "backbone." The NAG and NAMA molecules may also be linked together by β-1,3 and β-1,6 glycosidic bonds. Each row is composed of 10 to 65 amino sugars.

Each NAMA molecule is attached to a short tetrapeptide side chain of the amino acids and amino acid derivatives L-alanine, D-glutamic acid, diaminopimelate (DAP) and Dalanine. Cross linking peptide bonds between DAP and D-alanine in different linear sugar chains form a two-dimensional grid that is strong and rigid, although the exact amino acid sequence and overall structure varies within each bacterial species (Tortora *et al.,* 1986; Proctor and Cunningham, 1988). The structural formulas for NAG and NAMA with its amino acid side chains and linking is shown in Figure 6.

The cell wall in most Gram-positive bacteria is composed of multiple layers of peptidoglycan and is significantly thicker than that of Gram-negative bacteria.

Peptidoglycan of Gram-positive bacteria may comprise as much as 90% of the cell wall compared to as little as 5-10% in some Gram-negative bacteria (Withholt et al*.,* 1976; Prescott et al., 1999). The degree of cross-linking between adjacent peptides also varies between organisms. Some Gram-positive organisms may have close to 100% cross-bridging between peptides. In contrast, the frequency of cross-linking in *E. coli* (a Gram negative bacterium) may be as low as 30% resulting in more of a gel-like structure, rather than a more rigid, compact layer. Gram positive cell walls usually contain large amounts of teichoic acids, polymers of glycerol or ribitol joined by phosphate groups. Teichoic acids appear to extend to the surface of the peptidoglycan and help give the Gram positive cell wall its negative charge. These molecules may be important in maintaining the structure of the wall, however the exact function is still unclear (Prescott et al., 1999).

1.8.2 Outer Membrane and Lipopolysaccharide Layer:

Apart from differences in the peptidoglycan, Gram-negative bacteria have two unique regions that surround the outer plasma membrane: the periplasmic space and the lipopolysaccharide (LPS) layer. The periplasmic space separates the outer plasma membrane from the peptidoglycan layer and contains periplasmic enzymes and other proteins that participate in nutrient acquisition (i.e. hydrolytic enzymes attacking nucleic acids and phosphorylated molecules, and binding proteins involved in transport of material into the cell). The periplasmic space also contains enzymes involved in peptidoglycan synthesis and modification of toxic compounds that could harm the cell.

Electron micrographs have identified a similar but small periplasmic space in some Gram-positive bacteria, but do not appear to have as many periplasmic proteins (Prescott et al*.,* 1999).

The outer membrane is a protective barrier of Gram-negative bacteria and lies outside the thin peptidoglycan layer. It serves in preventing/slowing the entry of toxic substances (i.e. bile salts, antibiotics) that might kill or injure the bacterium. The LPS layer constitutes the majority of the outer membrane and is located adjacent to the exterior peptidoglycan layer. It is a complex phospholipid bilayer containing both lipid and carbohydrate consisting of three parts: (1) lipid A, (2) the core polysaccharide and (3) the O side chain (antigen). A small lipoprotein, Braun's lipoprotein, is covalently bound to the underlying peptidoglycan and embedded in the outer membrane by its hydrophobic end. Although complex, the outer membrane is not impermeable and can permit the passage of small molecules (ca. 600-700 daltons) through porin proteins which cluster together and span the outer membrane.

Adjacent LPS molecules of Gram-negative bacteria are also stabilized by the presence of divalent cations (i.e. Mg^{2+}, Ca^{2+}) in the outer membrane, which decrease electrostatic repulsions and increase LPS-LPS associations. These strong associations are believed to be the primary reason large hydrophilic molecules and most hydrophobic and amphiphilic molecules are prevented from gaining access to the cell (Nikaido and Nakae, 1979; Nikaido and Vaara, 1987). Alterations to the LPS, including removal of the divalent cations that stabilize the outer membrane, result in compromised functioning of this lipid bilayer barrier (Vaara, 1992).
The LPS is important for several reasons. The core polysaccharide usually contains charged sugars and phosphates which contribute to the negative charge on the bacterial surface, which along with the O antigen, aids the bacteria in avoiding various defenses of the host organism. Lipid A is a major constituent of the outer membrane and helps stabilize membrane structure. Additionally, lipid A often is toxic. As a result, the LPS can act as an endotoxin and cause some of the symptoms that arise in Gram-negative bacterial infections (Prescott et al., 1999).

1.8.3 Outer membrane integrity
Access to intracellular organelles or components of Gram-negative bacterial cells by many antimicrobials is limited due to the outer membrane (OM) which serves as an efficient outer permeability barrier against macromolecules. However, there is a propensity for strong interactions between the OM with highly cationic molecules because of the predominantly anionic nature of lipopolysaccharides and membrane proteins on the OM of Gram-negative microorganisms (Nikaido, 1996). Several cationic molecules have been shown to disrupt the integrity of the OM, resulting in loss of the barrier function without exhibiting direct bactericidal activity. The compounds such as polyethyleneimine (PEI) and polymyxin B nonapeptide (PMBN) bind to and functionally weaken the OM of gram-negative bacteria, sensitizing the organism to induced lysis or penetration by detergents, hydrophobic antibiotics or probes (Helander et al., 1997; Vaara and Vaara, 1983).
Electron micrographs have shown considerable structural alterations caused by the binding of these agents to the OM (Vaara and Vaara, 1983; Helander et al., 1998a; Helander et al., 2001). Vaara and Vaara (1983) also showed that other polycationic substances (protamine and certain polylysines) are able to disrupt the OM and simultaneously release major portions of LPS from the cells. EDTA has also been shown to release portions of LPS. This action is attributed to its metal-chelating action and removal of divalent cations that are essential for OM stabilization (Hukari et al., 1986). These substances, which lack inherent toxicity while increasing the OM permeability by various mechanisms, are termed permeabilizers (Vaara and Vaara, 1983). There lies significant potential for food-grade permeabilizers with application to food protection in that they would sensitize pathogenic Gram-negative bacteria to other inhibitory hurdles (e.g., heat, pressure, or other substances) by disrupting the OM barrier.

For example, Hughey and Johnson (1987) demonstrated that the resistance of Gram negative bacteria to lysozyme was diminished when the OM had been disrupted by EDTA.

Located beneath the cell wall (OM in Gram-negative bacteria), the cytoplasmic or inner membrane (IM) lacks the integrity of the peptidoglycan and OM. A delicate and semipermeable lipoprotein, it is responsible for controlling the entry and exit of solutes from the interior of the cell. Damage to this membrane (through membrane-active chemicals or physical processes such as high temperature and freezing) greatly affects the bacteria (Russell, 1998). Damage to this membrane can be detected very easily by measuring the extent of intracellular leakage (K+ ions, 260 nm-absorbing materials, nucleotides, denatured proteins, amino acids) from heated cells (Russell and Harries, 1968; Allwood and Russell, 1970; Tomlins and Ordal, 1970, Beuchat, 1978).

1.8.4 High Pressure Processing

High hydrostatic pressure (HHP) processed foods have been commercially available since 1990 in Japan. In 1996, the availability of these foods spread to Europe and the United States (Knorr et al., 1998). Compared with heat treatments, high pressure processing offers major benefits to the food and pharmaceutical industry. Because of the pressure range used for biotechnological interest, there is virtually no effect on covalent bonds. As a result, natural compounds such as flavors, aromas, dyes and pharmacologically active molecules are not adversely impacted (Smelt, 1998).

High pressure causes inactivation of vegetative microorganisms through membrane modification, inactivation of key enzymes, and inhibition of protein biosyntheses (Abe et al., 1999). Microbial growth is generally inhibited at pressures of 20–130 MPa and cell death occurs in the range of 130–800 MPa. Tolerance to high pressures varies according to the species, strain and suspending mediums used (Abee and Wouters, 1999).

Although the exact mechanisms of cellular damage by HHP have not been elucidated, moderate pressure may result in sublethal injuries of bacteria. As pressure increases, cellular membrane damage becomes more severe and coincides with a rapid increase in the death rate (Kalchayanand et al., 1998). (Abee and Wounters, 1999; Linton and Patterson, 2000; and De Angelis and Gobbetti, 2004).

1.8.8. Lysozyme

The Commission on Enzymes has assigned the classification numbers 3.2.1.17 to lysozyme. C-type (chick type) lysozymes are a homologous family of bacteriolytic enzymes which catalyze the hydrolysis of β-1,4-glycosidic bonds of polysaccharides, thereby compromising the peptidoglycan layer of bacterial cell walls and polymers of *N*acetyl- D-glucosamine (Jolles and Jolles, 1984). They are present in a variety of species ranging from microorganisms to invertebrates, to mammals.

Lysozyme was first discovered in 1922 by Alexander Fleming. While suffering from a cold, he found that his nasal mucous dissolved bacteria on agar plates. He soon discovered that the antibacterial action was due to an enzyme, but that it was only effective against certain bacteria and not those most infectious to man (Phillips, 1966). In 1963, Jolles and colleagues at the University of Paris and Canfield at the Columbia University College of Physicians and Surgeons discovered the chemical make-up of hen egg white lysozyme and mapped the single polypeptide chain sequence comprised of 129 amino acid residues. Lysozyme is linked in four places by disulfide bridges between cystine residues 64 and 80, 76 and 94, 6 and 127, and 30 and 115. Lysozyme hydrolyzes a number of substrates, but particularly the alternating polysaccharide copolymers of Nacetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) which represent the basic unit polysaccharide structure (peptidoglycan) of many bacterial cell walls. Lysozyme cleaves the β(1-4) glycosidic linkage connecting the C1 carbon of NAM to the C4 carbon of NAG. Tranter (1994) discovered that at least two of the disulfide bonds must be

intact for lysozyme to maintain its biological activity, and reduction of all four bonds results in a loss of all activity.

Jolles and Jolles (1984) sequenced hen egg white lysozyme and analyzed the three-dimensional structure by X-ray crystallography. It is an ellipsoidal molecule (45 x 30 x 30 Å) with two globular lobes (residue 5-36 and 98-129 comprising the first lobe and residues 40-94 comprising the second). A deep cleft lies between the two lobes which is able to host six hexose units (subsites one to six) held in place by hydrogen bonds and hydrophobic interactions. Amino acid residues are thought to contribute significantly to the specific binding of substrates. The optimal substrate is a (NAGNAMA) 3 hexasaccharide, with the active site of lysozyme including binding sites for each sugar ring of the hexasaccharide, these being designated sites A through F. When

the fourth hexose (D) in the chain becomes twisted out of its normal position (Figure 7a), a strain is imposed on the C-O bond on the ring-4 side of the oxygen bridge between rings D (NAMA) and E (NAG). Lysozyme's mechanism of action involves residue 35, glutamic acid (Glu-35), which is about 3Å from the -O- bridge that is to be broken. The free carboxyl group of glutamic acid is a hydrogen ion donor and transfers a hydrogen ion to the oxygen atom, breaking the already-strained bond between the oxygen atom and the carbon atom of the forth hexose (Figure 7b). This carbon atom acquires a positive charge that in turn temporarily attracts the negatively-charged carboxyl ion of residue 52, aspartic acid (Asp-52) which stabilizes the structure long enough for an -OH ion (from a spontaneously dissociated water molecule) to unite with the carbon (Figure 7c). The hydrogen ion (H+) remaining from the dissociated water can replace that lost by Glu-35 at which point the polysaccharide is broken and the enzyme is free to attach to a new location on the bacterial cell wall and continue its digestion activity.

Generally speaking, endo-N-acetylmuramidases, such as lysozyme, hydrolyze the glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine, resulting in the release of N acetylmuramic acid residues at the reducing end. Endo-Nacetylglucosaminidases, such as β-N-acetylglucosaminidase, hydrolyze the glycosidic bonds between N-acetylglucosamine and N-acetylmuramic acid, releasing fragments with N-acetylglucosamine at the reducing end (Ghuysen, 1968).

1.8.8.1 Bacterial Sensitivity to Lysozyme

As previously mentioned, lysozyme hydrolyzes the alternating polysaccharide copolymers of N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) of the peptidoglycan of many bacterial cell walls. Due to the absence of a lipopolysaccharide layer (LPS), which inhibits lysozyme accessibility, Gram-positive bacteria are more susceptible to the lytic activity of lysozyme. Salton and Pavlik (1960) tested various Gram-positive bacteria to determine their degree of susceptibility to lysozyme. Strains of *Bacillus, Corynebacterium, Lactobacillus, Micrococcus, Sarcina, Sporosarcina, Staphylococcus,* and *Streptococcus* were incubated for several hours with 50 µg of eggwhite lysozyme per ml of solution. Although all showed some degree of sensitivity to digestion with lysozyme, the percent reductions in populations varied from 9% for the most resistant (*Staphylococcus aureus*) cell walls to 98% for the most sensitive (*Micrococcus lysodeikticus*). The difference in lysozyme sensitivity may be attributed to a variance in β-glycosidic bonds present in the peptidoglycan of the various microorganisms.

Lysozyme primarily affects the β-1,4-N-acetylhexosaminidase bond, however cell walls may be composed of not only β-1,4 glycosidic bonds but also 1,3 and 1,6 bonds as well. The degree of sensitivity would relate directly to the proportion of β-1,4 glycosidic bonds present in the cell walls. Therefore, Gram-positive bacteria with higher β-1,3 and 1,6 glycosidic bonds would exhibit greater resistance to the action of lysozyme (Salton and Pavlik, 1960; Vakil et al., 1969).

Gram-negative bacteria are generally more resistant to lysozyme unless the LPS layer is disrupted by physical or chemical means. Treatments such as 2-amino-2-hydroxymethylpropane-1,3-diol (Tris) and ethylenediaminetetraacetate (EDTA) (Leive,1968) bind ions (i.e. Ca2+, Mg2+) that are essential for maintaining the integrity of the LPS layer. Antibiotics such as polymyxin B and aminoglycosides can also bind to the LPS and result in disruption of the outer layers of the cell. Additionally, shifts in the pH, osmotic shock (Birdsel and Cota-Robles, 1967), freeze-thaw cycling (Ray et al., 1984) and drying (Webb, 1969) can predispose the bacterial cell to the lytic action of lysozyme.

Peterson and Hartsell (1955) evaluated 135 Gram-negative bacterial species for sensitivity to lysozyme and concluded that only under extreme conditions [exposure to dilutions of hydrochloric acid (pH 3.5, 45°C, 1 hr) followed by adjustment to pH 9.8 with 0.05 N sodium hydroxide] does lysozyme act on Gram-negative cells. The degree of lysis towards each species varied from sensitive (*Salmonella, Brucella*), moderately sensitive (*Klebsiella, Shigella, Neisseria, Pseudomonas, Pasteurella, Erwinia, Escherichia*) to insensitive (*Vibrio, Proteus*). Hughey and Johnson (1987) evaluated lysozyme against food pathogens and spoilage bacteria and showed certain strains of C.*botulinium, C. thermosaccharolyticum, C. tyrobutyricum, Bacillus stearothermophilus* and *Listeria monocytogenes* were effectively inhibited or lysed by lysozyme. Lysozyme activity was also influenced by the presence of chelating agents (EDTA), as well as environmental conditions such as temperature, growth phase and medium. However, regardless of experimental conditions, some bacteria evaluated were resistant.

1.8.6. β-N-acetylglucosaminidase

β-N-acetylglucosaminidase is a ubiquitous glycocosidase exhibiting hydrolase activity against mucopolysaccharides, mucoproteins, glycoproteins and other mucosubstances (Levvy and Conchie, 1966). It is generally considered to be a lysosomal enzyme and attacks oligosaccharides derived from hyaluronic acid effectively removing terminal nonreducing N-acetylglucosamine residues (Weissmann *et al.,* 1964). β-Nacetylglucosaminidase is ubiquitous and found in animals (i.e., boar epididymis, hen ovoduct), plant tissues (i.e., jack bean), produced in prokaryotic organisms (i.e., *Lactococcus lactis, Clostridium perfringens*) and found in humans.

Bacteria synthesize peptidoglycan hydrolases capable of hydrolyzing their own peptidoglycan (Schockman and Höltje, 1994). These hydrolases are synthesized during cellular growth and are involved in a number of cellular functions that occur in cell wall remodeling such as cell separation after division, cell wall turnover and cell wall expansion (Smith *et al.,* 2000). N-acetylglucosaminidase is one of four different genera involved in cleaving the chemical bonds inside the peptidoglycan molecule. It is assumed to contribute to the hydrolytic degradation of glycoproteins, mucopolysaccharides and glycolipids (Robinson and Stirling, 1968). Very few studies exploring its antimicrobial activity have been initiated, however Martin and Kemper (1970) reported N-acetylglucosaminidase from *Clostridium perfringens* to be lytic against some Gram-negative bacteria, Kaplan et al. (2003) identified a soluble β-*N*-acetylglucosaminidase that causes detachment and dispersion of *Actinobacillus actinomycetemcomitans* biofilm cells, and Huard et al. (2003) identified an N-acetylglucosaminidase from *Lactococcus lactis* that exhibited hydrolyzing activity on the peptidoglycan layer of several Gram-positive bacteria.

1.8.6.1 β-N-acetylglucosaminidase from Hen Eggs

Lush and Conchie (1966) first identified the presence of β-Nacetylglucosaminidase (EC 3.2.1.30) activity in hen's egg white. They reported two know carbohydrases which occur in hen

egg albumen, β-N-acetylglucosaminidase and lysozyme. They found β-N-acetylglucosaminidase to be present in all hen egg albumen samples (n=39) from six strains of hens ranging in concentraitons from 2320 to 8880 µg (expressed as µg p-nitrophenol released from phenyl N-acetyl-β-glucosaminide by 1-ml albumen in 1-hr at 37°C). They also observed variations in the level of activity across the three main layers of egg albumen.

Several studies have reported varying concentrations of β-Nacetylglucosaminidase found in pooled fresh chicken eggs. Lush and Conchie (1966) first reported a 2-fold variation in the average concentration of β-Nacetylglucosaminidase isolated from 6 to 9 hens from 6 layer strains including Thornbergs 606, Sterling White Link, Brown Leghorn, White Leghorn (strains A and B) and Rhode Island Red. Henderson and Robinson (1969) later reported up to a 6-fold difference in the β-N-acetylglucosaminidase activity detected in samples of fresh egg white, whereas Donovan and Hansen (1971a) observed a 3-fold variation in enzymatic activity across individual fresh eggs. Their study followed the release of p-nitrophenol from p-nitrophenyl-N acetyl-β-D-glucosaminide using a modified procedure of Lush and Conchie (1966). The β-N-acetylglucosaminidase activity in pooled egg white from 15 different lots of egg albumen had a mean value of 4.12 mµmoles/min (expressed as pnitrophenol released from p-nitrophenyl-N-acetyl-β-D-glucosaminide by 0.01-ml albumen in 25-min at 37°C) with a standard deviation of 0.50 mµmoles/min. No seasonal variations in activity were observed.

Tarentino and Maley (1971) purified two isozymes of β-N-acetylglucosaminidase from hen oviduct with reported molecular weights of 118,000 (Type I) and 158,000 daltons (Type II) and p values of 6.45 and 6.86, respectively. Kinetic constants and activity towards various substrates exhibited virtually no differences between the two.

However, the Type I enzyme is the predominant species. Lucas (1979) later showed that only Type I isozyme is found in egg white and suggested that it may play a role in the catabolism of glycoproteins in both egg white and the oviduct. Contrary to the findings of Tarentino and Maley reported above, Ogawa and Nakamura (1983) and Ogawa *et al.,* (1983) also purified a β-*N*-acetylhexosaminidase (NAHase) from both hen oviduct and egg white, and then chicken liver and chicken serum. Oviduct and liver NAHase was reported to have a similar MW of 53,000, while serum and egg white NAHase had a MW of 68,000. They hypothesized that these variations could be because lysosomal enzymes may be synthesized as precursors with a subunit molecular weight higher than those of mature forms.

1.8.6.2 β-N-acetylglucosaminidase Activity

β-N-acetylglucosaminidase activity in egg white responds negatively to increases in both temperature and pH. There is gradual loss in its activity as the pH exceeds especially at ambient temperatures. Lush and Conchie (1966) evaluated albumin β-Nacetylglucosaminidase activity in eggs stored at room temperature. They observed a rapid decline in enzymatic activity from a mean of 3283 units to 250 units after storage for 10 days and then decreased to 30 units after 19 days (expressed as µg p-nitrophenol released by 1-ml albumen in one hour at 37°C). They also found that the enzyme activity was not affected by freezing/thawing or homogenization of the albumen. Donovan and Hansen (1971a) showed that when refrigerated, the enzyme activity in fresh eggs remained high for a considerable length of time. Winn and Ball (1975) reported that the loss of β-N-acetylglucosaminidase activity is closely associated to the increase in albumen pH. They recorded a rapid decline in enzyme activity to near extinction between 3 to 6 days of age at pH 9.2 to 9.4 when eggs were stored in open flats at 25°C.

In contrast, β-N-acetylglucosaminidase in shell membrane was found to be very stable. Winn and Ball (1975) reported that, for eggs stored for nine days at room temperature, activity in the membrane was not affected by pH changes in the surrounding albumen (which reached pH values of over 9.0). They proposed that the enzyme might be protected as a component of the membrane. They further suggested that if β-Nacetylglucosaminidase is an integral part of the shell membrane, it might play a bacteriostatic role or assist in slowing bacterial penetration into the egg.

The optimum pH for β-N-acetylglucosaminidase activity varies depending upon the source of the enzyme. Donovan and Hansen (1971a) determined the optimum pH of hen albumen β-N-acetylglucosaminidase activity to be 3.0 with at least 20% of maximum activity observed over a pH range of 1.5 to 5.5. The enzyme activity in egg albumen (from pH 6.8 to 8.8) was stable for several hours at ambient temperature (24°C). When the egg albumen to pH was increased to 9.6 with 1 M NaOH, the enzyme activity was rapidly lost.

Henderson and Robinson (1969) were the first to report that β-Nacetylglucosaminidase from hen albumen is heat inactivated when exposed to temperatures near 60°C. They determined the activation energy for heat denaturation in egg albumen (no pH specified) to be 62.9 kcal/mole. Later, Donovan and Hansen (1971b) demonstrated that the kinetics of β-Nacetylglucosaminidase heat inactivation in egg white and whole egg (pH 7.0) followed a first order rate when heated between 58-62°C and an activation energy of 91 kcal/mole in egg albumen and 73 kcal/mole in whole egg. At 60°C (pH 7.0), approximately 66% of the enzyme activity was destroyed by heating for 3.5 min. To date, there has been no published literature pertaining to the antimicrobial activity of hen β-NAGase.

1.9.1 Ovotransferrin

Ovotransferrin, also referred to as conalbumin, constitutes about 13% of egg white proteins. It belongs to the transferrin family and shows about 50% sequence homology with mammalian serum transferrin and lactoferrin. It is a glycoprotein consisting of 686 amino acid residues with 15 disulfide bridges, a MW of approximately 77,700 and an isoelectric point (pI) of 6.1-6.5 (Abola *et al.*, 1982). It is folded into two lobes referred to as the N- and C-lobe. Although similar in structure, they differ functionally. The two lobes are linked together with a short connecting peptide and each lobe can be divided into two domains enclosing a hydrophilic cleft which acts as a transitional metal (i.e. (Fe[III], Cu[III], Al[III]) binding site.

1.9.2 Action of Ovotransferrin on Bacteria

It was believed the antimicrobial activity exhibited in ovotransferrin was only a result of iron deprivation (iron being an essential growth factor for most microorganisms) due to the sequestered iron in the binding site (Alderton, Ward and Fevold, 1946). The antimicrobial activity of ovotransferrin (Valenti *et al.*, 1983) against a variety of microorganisms, including pathogenic *Escherichia coli, Pseudomonas aeruginosa* and *Vibrio cholera*, has been demonstrated (Boesman-Finkelstein and Finkelstein, 1985). *In vivo*, ovotransferrin has been shown to have therapeutic properties against acute enteritis in infants (Corda *et al.,* 1983). Several reports of ovotransferrin retaining antimicrobial activity, even when complexed with metals such as zinc and iron, have been reported (Valenti et al., 1987; Valenti et al., 1985; Ibrahim, 1996).

Structural similarity between lactoferrin and ovotransferrin exist, however evidence suggests that ovotransferrin possesses structurally-dependent bactericidal activity other than the iron deprivation effect reported for lactoferrin. Regardless of the degree of iron saturation, ovotransferrin exhibited strong antimicrobial action against Gram-positive *Staphylococcus aureus*. For the Gram-negative organism *Escherichia coli*, the iron-bound ovotransferrin was more bactericidal than the iron-free ovotransferrin suggesting that the mechanism of action is attributed to factors other than the iron deprivation effect (Ibrahim et al., 1998). Aguilera and collegues (2003) demonstrated the ability of ovotransferrin to permeate the outer membrane of *Escherichia coli*. This interaction was attributed to the anionic nature of the outer membrane and the cationic nature of ovotransferrin. Although ovotransferrin was not able to cause extensive damage to the bacteria, there was a marked increase in extracellular [K+] concentrations, without detectable changes in [Na+]. A dissipation of the electrical potential was observed along with increased sensitivity of bacterial cells to the hydrophobic antibiotic, actinomycin D. Actinomycin D, which normally possesses limited action against Gramnegative bacteria, inhibited RNA and protein synthesis upon disruption of the outer membrane.

1.10.. Lethal Effects of Heat on Bacteria:

Temperature response varies between bacteria, including the lethal effects of high temperatures. Non-sporulating bacteria are readily inactivated at temperatures of about 50° C and above. Generally, the rate of inactivation increases as the temperature is raised (Russell, 1998). "Several factors, such as composition and pH of the menstruum, the type of organism (there could be a strain-dependent response), the growth conditions, heating method and recovery conditions influence the rate of bacterial inactivation (Russell, 2003)." Most cellular component are likely affected to some degree by high temperatures (e.g., the denaturation or coagulation of proteins, breaks in DNA, lesions in RNA and damage to the outer layers/membranes) and have been suggested as possibilities as to the lethal affect in thermal inactivation of microorganisms.

Early thought prescribed that the denaturation or coagulation of proteins involved in cell respiration or cell multiplication was the cause of death (Banwart, 1979). At temperatures ranging from 50° to 60° C, leakage of cellular components into the suspending medium indicates damage to the permeability of the cell. However, at higher temperatures, death can precede leakage. Scheie and Ehrenspeck (1973) suggested that heat caused a denaturation of proteins in the cell envelope of *E. coli,* thus weakening the peptidoglycan layer sufficiently to prevent multiplication. Further injury results from internal osmotic pressure which ruptures the cell membrane at weakened areas. Banwart (1973) assumed that destruction of cells at temperatures that cause sublethal injury may involve the cell membrane. However, if death precedes leakage at higher temperatures, other mechanisms must be involved. Russell and Harries (1968) reported "that for nonsporeforming cells, such as *E. coli*, RNA degradation is closely related to heat induced death. Protein coagulation, if not the primary lethal event, is important in the thermal destruction of microorganisms, as denaturation of proteins can occur in several areas ofthe cell."

Literature Review

2.1 Chemical Composition :

This study was undertaken to determine the occurrence of uronic acid in chicken eggshell membranes and to compare chemical compositions among the inner and outer eggshell membranes and the organic matter of eggshell. We report here for the first time the occurrence of uronic acid in chicken eggshell membranes. Uronic acid concentrations were similar (P >0.05) between the inner shell membrane and outer shell membrane but approximately fivefold higher (P >0.05) in the organic matter of eggshell. Sialic acid concentrations werethe highest (P <0.05) in the organic matter of eggshell and higher (P >0.05) in the inner than in the outer shell membrane. Nitrogen concentrations were the lowest (P >0.05) in the organic matter of eggshell but relatively constant between the two shell membranes. Amino acid analysis showed that the contents of glycine and alanine
were higher (P >0.05) and those of proline and hydroxyproline were lower (P ⬜0.05) in the organic matter of eggshell compared to shell membranes.(2003 Poultry Science 82:510–514)

2.2: Utilization of Eggshell Powder as Excipient:

Eggshell powder has been investigated for the new application as pharmaceutical excipient in tablet dosage form. Acetaminophen was used as a model drug in this study. Four different eggshell powders were prepared. These included untreated eggshell powder, water treated, ethanol treated and chloroform treated eggshell powders. The treated samples were prepared by surface modification using 1.0 % w/v stearic acid in solvent namely deionized water, 95 % ethanol and chloroform. The tablets containing acetaminophen, eggshell powder and microcrystalline cellulose were prepared by direct compression method. Dissolution studies of four acetaminophen formulations in pH 5.8 phosphate buffer were performed using USP Dissolution Apparatus II. The results show that immediate release of acetaminophen was obtained from tablets containing untreated eggshell powder whereas sustained release of the drug was obtained from the tablet formulations containing three different treated eggshell powders. Sustained release of the drug may be due to hydrophobic nature of the treated eggshell powders. It was also found that the degree of hydrophobicity of the treated eggshell powders depends on the type of solvent used in surface modification process. The results obtained from this study show that eggshell powder appears to be applicable as a pharmaceutical excipient to control the drug release from the tablet. Additionally, this finding may be useful to generate public interest in development of biomaterials from eggshell waste by proposing the new application of eggshell powder in pharmaceutical industry.
(Nature, 1988;336:699-700.)

2.3 Antibacterial Properties of an Egg:

Reports of outbreaks of salmonella enteritidis food poisoning associated with consumption of hen eggs or egg products have appeared with increasing frequency all over the world1-3. The main causative organism was salmonella enteritidis phage type IV. Spread of infection was from an important new source, contents of intacthen eggs. The proportion of eggs that are infected internally is very low indeed but because millions of eggs are consumed daily the number of human infections represent an important public health problem. In the United States of America, late in 1986, veterinary investigators demonstrated that trans-ovarian infection with salmonella enteritidis phage type VIII can lead to human food poisoning from shell eggs3. As eggs are a major portion of every day diet, there are many studies on the process of Infection of shell egg

and its natural defence systems. The first systematic investigation on microbial deterioration of eggs intended for human consumption was conducted by Gayon in 18734. Haines In 1939 stated that egg is equipped with physical and chemical defence against microbial infection and suggested that, these have evolved to protect the embryo during. incubation4.

2.4 Antimicrobial properties of avian eggshell:

C-type lectin-like proteins are major components of the calcified eggshell of multiple avian species. In this study, two representative avian C-type lectin-like proteins, ovocleidin- 17 and ansocalcin, were purified from decalcified chicken and goose eggshell protein extracts and investigated for carbohydrate binding activity as well as antimicrobial activity. Purified ovocleidin- 17 and ansocalcin were found to bind bacterial polysaccharides, and were bactericidal against Bacillus subtilis, Staphylococcus aureus and Pseudomona aeruginosa. Bactericidal activity was found to be enhanced in the presence of calcium but was not dependent on its presence. The results suggest that avian C-type lectin-like proteins may play an important antimicrobial role in defence of the avian embryo. to the Reg family of C-type lectins. Given their widespread presence and abundance within the eggshell matrices of various species, we sought to investigate the antimicrobial properties of avian eggshell CTL proteins. Our strategy was to study the binding of ansocalcin and OC-17 to bacterial cell wall polysaccharides, and investigate their antimicrobial properties against Gram-positive and Gram-negative bacteria. The results show that avian eggshell CTL proteins have excellent antimicrobial as well as bacterial polysaccharide binding properties. This is the first report on the antimicrobial properties of eggshell-specific CTL proteins and highlights the multiple roles played by these proteins. (Peter Brzezinski,et al, 2008)

2.5 ANTIMICROBIAL ACTIVITY:

In another study showed that,eggshell membrane bound components were capable of reducing the heat resistance and/or inhibiting the growth of selected Gram-positive and Gram-negative foodborne bacterial pathogens suspended in 0.1% peptone water (Poland and Sheldon, 2001). More specifically, 83 - 87% reductions in thermal decimal reduction times (D-values) for *Salmonella enteric* serovars Typhimurium and Enteritidis (D54ºC), and *Escherichia coli* O157:H7 (D52ºC) were noted. Also, a 3 log reduction in the population of *L. monocytogenes* was observed following incubation for 45 min at 37ºC. The exact nature of the protein and non-protein constituents responsible for these antibacterial properties was not identified. Therefore, the focus of this study was designed to extract enzymatically and biologically active fractions of β-N-acetylglucosaminidase, lysozyme, and ovotransferrin in order to evaluate their function in providing ESM antimicrobial protection.

2.6 Enzymatic and microbiological inhibitory activity :

Eggshell membranes (ESM) have been shown to exhibit antibacterial activity. The purpose of this study was to evaluate the enzymatic and biological [decimal reduction times (D-values)] activities of ESM as a function of bird breed, age, and ESM stabilization treatments. Younger White Leghorn (WL) hens produced ESM with 28% higher lysozyme activity than Rhode Island Red (RIR) layers. In contrast, older WL layers produced ESM with 17% less lysozyme activity than ESM from RIR layers. Similarly, beta-N-acetylglucosaminidase (beta-NAGase) ESM activities differed by hen age within breeds with younger hens yielding 14 to 16% more enzyme activity. D54 degrees C-values of Salmonella Typhimurium cells preexposed to WL ESM did not

differ as a function of bird age (33, 50, and 81 wk). The ESM Lysozyme and beta-NAGase activities varied somewhat over a 6-mo storage study after treatment with 1 of 5 stabilization methods [i.e., storage at 4 degrees C, -20 degrees C, or ambient air storage after freeze drying, air drying (23 degrees C), or forced-air drying (50 degrees C)]. Both air and forced-air drying yielded significant reductions in beta-NAGase and lysozyme ESM activity (ca 12 to 30%) after the initial 24 h and then remained fairly stable during the extended storage. Freeze-dried samples retained the most enzymatic activity (95%) throughout the 6-mo trial, whereas refrigerated ESM lost 20 and 18% of the beta-NAGase and lysozyme activities, respectively. Frozen ESM lost 22% of the beta-NAGase activity, whereas lysozyme was nearly unaffected after 6 mo. The ESM biological activities against S. Typhimurium were not adversely impacted by layer breed or age. No significant loss in biological activity of ESM was detected 24 h after processing or after 6 mo of storage for refrigerated, frozen, and freeze-dried membranes, whereas significant reductions were observed for air- and heat-dried ESM. These findings demonstrate that ESM enzyme and biological activities are relatively constant across layer breeds and over extended storage. Based on these and other findings, ESM may have potential commercial value as a processing adjuvant in food and pharmaceutical product applications.(POLONIA ,VOL. LXIV (2) SECTIO DD 2009)

2.7 Anti-inflammatory activity of eggshell membranes:

The present invention relates to anti-inflammatory activity of eggshell membrane, processed eggshell membrane preparations and eggshell membrane isolates. The invention is directed to eggshell membrane compositions exhibiting anti-inflammatory activity as measured by effects on down-regulating pro-inflammatory plasma antigens in mammals that had orally ingested such compositions. This information supports the potential benefits from administration of naturally occurring material found in eggshell membrane, processed eggshell membrane, eggshell membrane isolates and combinations to reduce pain and inflammation associated with arthritis and other inflammatory conditions.

IDENTIFICATION AND PREPARATION OF SHELL

EXTRACT

3.1 SHELL COLLECTION:

Egg shell are collected from the resturent of naryangonj in janurary 2015.

3.2 DRYING OF SAMPLE:

After the collection of sample it needs to be dried to make the sample extract. In general the plant material should be driedat temperature below 30 degree C to avoid the inhibition of bacteria. So sun drying can be very effective but drawback is sometimes water molecules are absorbed by the sample and hence fungus growth can affect the study. The egg shell were dried in the sun light thus chemical decomposition can not take place.

3.3 GRINDING OF DRIED SAMPLE:

Small amount of plant material can be milled using grinder or blender. But if the sample is in high amount then it is easier to get powdered sample by grinding from a spice mill. Grinding improves the efficiency of extraction by increasing surface area. It also decreases the amount of solvent requiredfor the extraction. The dried samples were ground to coarse powder with a mechanical grinder (Blender) and powdered samples were kept in clean closed containers pending extraction. During grinding of samples, the grinder was throughly cleaned to avoid contamination with any remnant of previously ground material or other foreign matter deposit the grinder.

3.4 MACERATION OF DRIED POWDERED SAMPLE

3.4.1 Principle

The choice of extraction procedure depends on the nature of the plant material and the components to be isolated. The principle of solid-liquid extraction is that when a solid material comes in contact with a solvent, the soluble components in the solid material move to the solvent. Thus, solvent extraction of sample material results in the mass transfer of soluble active principle (medicinal ingredient) to the solvent, and this takes place in a concentration gradient.The rate of mass transfer decreases as the concentration of active principle in the

solvent increases, until equlibrium is reached, i.e, the concentration of active principle in the solid material and the solvent are the same. Thereafter, there will no longer be a mass transfer of the active principle from sample material to the solvent. Since mass transfer of the active principle also depends on its solubility in the solvent, heating the solvent can enhances the mass transfer.Moreover, if the solvent in equilibrium with the sample material is replaced with fresh solvent, the concentration gradient is changed.

3.4.2 Procedure

After getting the sample as dried powdered, the sample (1280 Gram) was then soaked in 2000 ml of methanol for 5 days, the process is known as maceration technique.A glass made jar with plastic cover was taken and washed throughly with ethanol and dried. Then the dried powder sample was taken in the jar. After that methanol (1000 ml) was poured into the jar up to 1-inch height avobe the sample surface as it can sufficiently cover the sample surface. The plastic cover with aluminium foil was closed properly to resist the entrance of air into the jar.This process was performed for 5 days. The jar was shaked in several times during the process to get better extraction.

3.5 FILTRATION OF THE EXTRACT

After the extraction process the sample extracts was filtered with sterilized cotton filter.The cotton was rinsed with ethanol and fitted in a funnel.The filtrate was collected in a beaker.Then again it was filtered and this time What man's filter was used for getting more clear extract which would be useful making the sample more concentrate in Rotary Evaporation Technique.Then the filtrate was taken into a volumetric flask and covered with aluminium foil paper and was prepared for rotary evaporation.

3.6 SAMPLE CONCENTRATION BY ROTARY EVAPORATION TECHNIQUE

3.6.1 Principle

- A rotary evaporator is a device used in chemical laboratories for the efficient and gentle removal of solvents from samples by evaporation.Rotary evaporators are also used in

moleculer cooking for the preparation of distillates and extracts.A rotary evaporator consists of folllowing parts-

- A motor unit that rotates the evaporation flask or vial containing the user's sample.

- A vapor duct that is the axis for sample rotation, and is a vacuum-tight conduit for the vapor being drawn off of the sample.

- A vacuum system, to substantially reduce the pressure within the evaporator system.

- A heated fluid bath (generally water) to heat the sample.

- A condenser with either a coil passing coolant, or a "cold finger" into which coolant mixtures such as dry ice and acetone are placed.

- A condensate-collecting flask at the bottom of the condenser, to catch the distilling solvent after it re-condenses.

- A mechanical or motorized mechanisms to quickly lift the evaporation flask from the heating bath.

3.6.2 Affecting Factos

There are following factors, omission of one of the following factor may interfere the sample concentration procedure and thus which may interfere the phytochemical investigation.

- Remove the flask from the heat bath.

- Opening the stopcock.

- Heating the rotor.

- Turning off the vacumm/aspirator.

- Disconnecting the flask.

- Dropping flask in heat bath.

3.6.3 Procedure

After the filtration process two parts were obtained namely 'residue part' and 'filtered part or filtrate'.The filtrate part, which contains the substance soluble in methanol, was putted into a 1000 ml round bottom flask (BOROSOL) and then the flask was placed in a rotary evaporator.The evaporation was done at 45 degree Celsius temperature.The number of rotation per minute was selected as 130 RPM.The pressure of the vacuum pumper machine (Biometra) was 6 bar.The water flow through the distillation chamber was also provided in a satisfactory flow rate.When the evaporation seemed to be satisfactory, then the methanolic extract was collected in a 100 mi beaker.The evaporator flask was rinsed by diethyl ether. Then the beaker was covered with aluminium foil paper and kept on the water bath for 60 minutes and the temperature of water bath maintained as 50' C.Finally the concentrated methanolic extract was found and stored in the laboratory refrigirator from which the extract was used for many chemical investigation.

METHODS AND MATERIAL

4.1 THIN LAYER CHROMATOGRAPHY (TLC)

4.1.1 Principle

Thin layer chromatography is used to separate mixtures of compounds.It gives a qualitative idea about the components that are present in a mixture.The experiment is usuallly conducted on a sheet of aluminum foil that has been coated with silica.This acts as the stationery phase for the mixture.The solvent or solvent system that runs on the stationery phase by capillary action and conducts the separation, this is known as the mobile phase.Once the sample has been spotted on the plate and the mobile phase run through it, the different components of the mixture separate differently owing to their relative affinities for the stationery and mobile phases.Heavier components or the ones more attracted to the statoinery phase remain at the bottom while components that are light and more soluble in the mobile phase travel up with it.The relative separation of the components can be studied by calculating the Retardation Factor (Rf), which is the ratio of the distance of migration of a particular substance to the distance of migration of the solvent front.

4.1.2 Materials Required

- Silica coated TLC plate.

- TLC tank.

- Spotting capillary tubes.

- Tweezers.

- Pipette.

- Pipette filter.

- Test tubes.

- Solvents.

- UV lamp

4.1.3 Reagents

- Benzene.

- Ethanol.

- Ammonium Hydroxide.

- Chloroform.

- Ethyl Acetate.

- Formic Acid.

- Water.

- N-hexane.

- Di-chloromethane.

- N-butanol.

- Methanol.

Table 4.1.4 The composition of various solvent systems for TLC

Nonpolar Solvent		Intermediate polar Basic Solvent		Polar Basic Solvent
Benzene 9ml		Chloroform 5ml		Ethyl acetate 8ml
Ethanol 1ml		Ethyl acetate 4ml		Ethanol 1.2ml
Aloh 0.1ml		Formic acid 1ml		Water 0.8ml

4.1.4 Procedure

- Using a pencil the baseline and the solvent front line was drawn on the TLC plate and the plate was labeled for the individual spots.

- The fraction of methanolic extract was spotted on TLC plate and the plate was dried completely in the air.

- In a TLC tank the solvent system was added. Astrip of filter paper was inserted into the tank so that its bottom touched the solvent. The lid of the tank was closed and left to rest for a few minutes so that the solvent system could travel up the filtr paper and saturate the chamber.

- Using a pair of tweezers the TLC plates were placed in the chamber carefully so that the baseline did not touch the solvent.

- The plate was left in the tank so that the solvent system could run up the plate by capillary action and develop the spots.

- The plate was removed from the tank using a pair of tweezers once the solvent had reached reached the solvent line. The plate was then allowed to dry completely.

- Three types of solvent system were used based on difference in polarity for the detection of different compounds.

- The developed plate was then viewed under UV light for the detection of bands and spots.

4.1.5 Acid Charing of TLC plates

4.1.5.1 Materials

- Tweezers.

- Conc. Sulfuric acid.

- Distilled water.

- Hot plate.

- Petri dish.

4.1.5.2 Procedure

- 9 ml of distilled water was added to 1 ml of concentrated sulfuric acid to produce a 10% solution of sulfuric acid which was taken in a petri dish.

- The TLC plate was dipped in this solution using tweezers with the silica face down.

- The plate was left in the open for 10 minutes to allow for drying.

- A hot plate was heated to about 90 degree C and the plates were heated until the spots developed.

4.1.6 DPPH CHARRING PROCESS OF TLC PLATE

4.1.6.1 Materials Required

- 4% DPPH stock solution (1%), Methanol (9 ml).
- Test Tube.
- Pipette.
- Pipette filter.
- Petridish and Tweezers.

4.1.6.2 Procedure

- 0.4% solution of DPPH was prepared by adding 9 ml of methanol to 1 ml of 4% DPPH stock solution. The procedure was carried out in a dark room as DPPH is light sensitive.
- By using tweezers the developed TLC plates would be dipped into this solution on the silica face down.
- The plates were left in the dark room for 30 minutes for the color to develop after which they were observed for the formation of yellow, golden / brown color on the background of purple. This coloration indicates the presence of compounds that have antioxidant properties (Milena, N., 2011).

4.1.7 APPLICATION OF TLC TECHNIQUE

- Separation of components from a mixture based on their relative affinity towards the solvent system (mobile phase) and the stationary phase**.**
- To check compatibility of different excipients in a formulation**.**
- TLC is done on analytical scale as a means of monitoring the progress of a reaction**.**
- TLC is done on the preparative scale to identify and purify small amounts of a compound**.**
- 5)To detect any impurities (Milena et.al., 2011).

4.1.8 Disadvantages Of TLC technique

- TLC plates do not have long stationary phases. Therefore, the length of separation is limited compared to other chromatographic techniques.
- Only small amount of compound can be identified and purified (Milena et.al., 2011).

4.2. ANTI-OXIDANT TESTS

DPPH Test (1,1 -diphenyl-2-picrylhydrazyl radical)

4.2.1 Principle

The 1,1 -diphenyl-2-picrylhydrazyl radical (DPPH) has ben widely used to evaluate the free radical scavenging capacity of antioxidants.DPPH free radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors.DPPH can make stable free radical in aqueous or methanol solution.With this method it was possible to determine the antiradical power of an antioxidant activity by measurement of the decrease in the absorbance of DPPH at 517 nm (Prieto and Pineda, 1999).
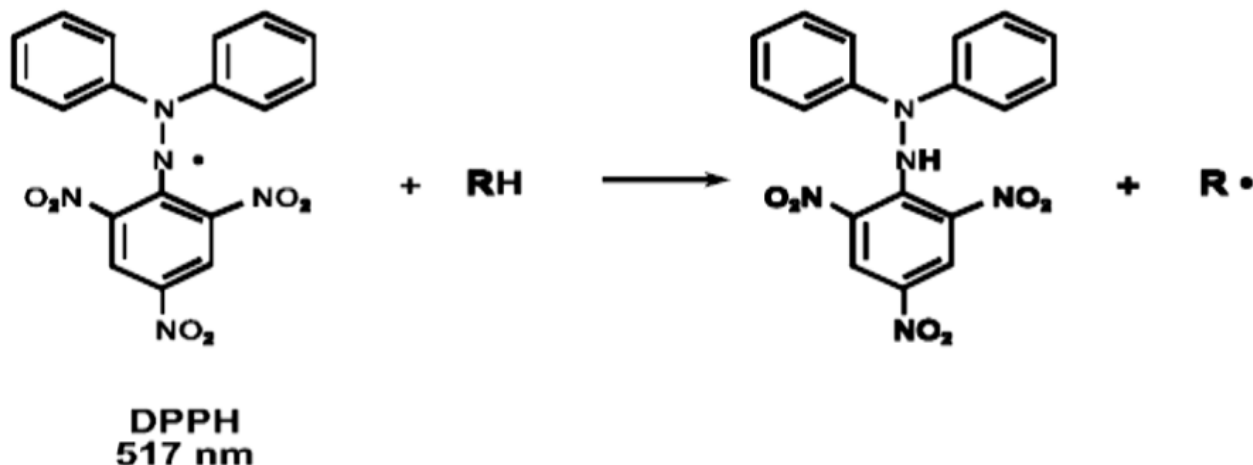
**DPPH
517 nm**

Fig 4.2.1 Chemical reduction of DPPH free radicals

The structure of DPPH and its chemical reduction is shown above. The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color. This molecule gets reduced to a yellow to colorless form when it takes up one hydrogen free radical from a freeradical scavenging antioxidant to form DPPH-H, which is a stable molecule.The presence of greater amounts of antioxidants in the sample causes a greater reduction of DPPH and this can be used to monitor antioxidant activity by increasing the amount of samples.With increasing concentration samples, The absorbance of DPPH at 517 nm drops. A blank is prepaed by adding DPPH to only the solvent and not the sample. The percentage antiradical scavenging activity is calculated by the following formula-

DPPH % antiradical scavenging activity = 1 – (Absorbance of sample / Absorbance of

Blank) × 100.

4.2.2 Apparatus

- Test tube.

- Racker.

- Beaker.

- Uv-spectrophotomete.r

- Spatula.

- Analytical balance.

4.2.3 Reagents

- DPPH.

- Methanol.

- Water.

4.2.4 Procedure

4.2.4.1 Sample Preparation

- The methanolic extract of the egg shell of different fraction were taken in test tubes to prepare different concentrations.

- 1µg/ml sample was taken in test tubes,and prepared 10 ml sample solution with 9 ml water. Then each sample was diluted ino 1ml, 2ml, 3ml, 4ml and volume adjusted to 4ml with water in all the test tubes.

4.6.1.7 Blank Preparation

Blank was prepared by adding 1 ml methanol in a test tube and volume adjusted with 9 ml water.Blank was made in same way of the sample.

- After preparation of sample and blank preparation 100 µl DPPH solution was added in dark and left for half an hour. After that UV absorbance was measured in UV machine at 517 nm.

- After 30 min at room temperature, the absorbance values were measured at 517 nm on a spectrophotometer and converted into the percentage antioxidant activity using the equation, DPPH antiradical scavenging capacity (%) = [1 – (Abof sample – Abof blank)/Abof control] × 100.

4.3 IN VITRO ANTI-DIABETIC TEST

4.3.1 Introduction

Diabetes mellitus, a metabolic endocrine disorder, has become a common global health problem that affects >170 million people worldwide. It is one of the leading causes of death and disability. It is estimated that by 2030, the number will rise to 366 million. The majority of diabetes (~90%) is type 2 diabetes (T2D) caused by a combination of impaired insulin secretion from pancreatic beta cells and insulin resistance of the peripheral target tissues, especially muscle and liver. Uncontrolled diabetes can complicate pregnancy and birth defects. Generally, the long-term complications diabetes are categorized into macrovascular (coronary artery disease, peripheral arterial disease, and stroke) and microvascular (diabetic nephropathy, neuropathy, and retinopathy) (Bastaki., 2005).High glucose level in the blood can be subjected for a spontaneous reaction that occurs between the aldehyde group and amino groups of the protein chain that leads to the formation of the Schiff base. This intermediate subsequently undergo a number of biochemical reactions to yield various fluorescent brown pigments known as advanced glycation end products (Esmaeili, et.al.,2010). The oxidation process is believed to play an important role in formation of ages. Further oxidation of ages leads to the formation of intermediate carbonyl compounds that can react with the nearby lysine or arginine residues to form protein crosslinks and therefore, agents with antioxidant property may retard the process of ages formation and their subsequent oxidation progression. Recently, role of fruits in the prevention of glycation activity has gained a substantial attention among scientific community and various families like Anarcardiaceae, Curcubitaceae, Moraceae, Annonaceae, Sapotaceae, Phyllanthus, Myrtaceae, Rutaceae etc. Have been documented for their noteworthy role to own antioxidant and antiglycation activities. Several natural compounds like curcumin, rutin and garcinol have been reported to possess antioxidant property as well their role in the prevention of formation of ages in vitro and in vivo (Jedsadayanmata,2005).The investigation of antidiabetic agents of plant origin which are used in traditional medicine is of great importance. The seed kernel of Mangifera indica is one such herbal source which is mentioned in Ayurvedic literature for treating Diabetes mellitus. The kernel is astringent, antihelmintic,stimulant, anti-inflammatory, antibacterial, antifungal, anti-pasmodic, anti-scorbutic and is administered in asthma, diabetes, nasal bleeding, diarrhea and ulcers (Jain, 2011).Similarly, Egg Shell possess numerous medicinal properties such as antibacterial, antioxidant, antidiabetic, anti-inflammatory, anti-diuretic, immunomodulatory.

Non-enzymatic reaction between reducing sugar and free amino group of proteins, also known as Maillard reaction, leads to the formation of glycated protein termed Amadori product. Further rearrangement, oxidation and reduction of the Amadori product result in the formation of several advanced glycation endproducts (ages) such as pentosidine, carboxymethyllysine, crossline and pyralline. Some of these products can react with a free amino group nearby and form crosslinking between proteins (Ulrich and Cerami, 2001). The crosslinked protein, e.g. Crosslinked collagen, are postulated to confer pathological conditions found in patients with diabetes and aging, such as arterial stiffness and decreased myocardial compliance, resulting from the loss of collagen elasticity (Aronson, 2003). Thus, agents that inhibit the formation of ages are purported to have therapeutic potentials in patients with diabetes and age-related diseases. The oxidation process is believed to play an important role in ages formation. Further oxidation of Amadori product leads to the formation of intermediate carbonyl compounds that can react with the nearby lysine or arginine residues to form protein crosslink and ages. The reactive carbonyl compounds may also be generated from the metal ion-catalyzed autooxidation of glucose (Voziyan et al., 2003).

4.3.2 Principle

Glycation is the key molecular basis of several diabetic complications like diabetes retinopathy, nephropathy, neuropathy and some cardiovascular diseases. This is a non enzymatic reaction between amino groups of proteins and carbonyl groups of reducing sugars forming florescent, insoluble Advanced Glycation End Products that accumulate on loprinciplglycation ng lived proteins thus compromising the physiological functions. The reaction is sub divided into three stages as early, intermediate and late. In early stage unstable Schiff base forms and through acid base catalysis this compound undergoes further rearrangement to a more stable Amadori product and via dehydration, oxidation and other chemical reactions degrades to more reactive carbonyl compounds and they act as propagators of the reaction again reacting with more free amino groups of biomolecules. In the late stage Advanced Glycation End Products (ages) are formed. The formation of ages progressively increases with normal aging and is accelerated in diabetes. Various studies have shown that diabetes mellitus is associated with an increased production of free radicals leading to oxidative stress. Thus disturbed balance between radical formation and radical neutralization leads to oxidative damage of cell components such as proteins, lipids and nucleic acids. Oxidation plays an important role in the formation of Advanced

Glycation End Products and the Plants derived agents with the antiglycation and antioxidant activities are highly important in preventing diabetic complication.

4.3.3 Procedure

Antidiabetic activity of egg shell were investigated by glucose uptake in yeast cell.Yeast cells were prepared according to the method of Yeast cells (Kotowaroo et.al.,2006). Briefly, commercial baker's yeast was washed by repeated centrifugation(3,000×g; 5 min) in distilled water until the supernatant fluids were clear and a 50% (v/v) suspension was prepared in distilled water. Various concentrations of Isolated constituents (1 mg) after VLC were added to 1 ml of glucose solution (10 mM) and incubated together for 10 min at 37 °C. Reaction was started by adding 100 µl of yeast suspension, vortex and further incubated at 37 °C for 60 min. After 60 min, the tubes were centrifuged (2,500 × g, 5 min) and glucose was estimated in the supernatant. Metronidazole was taken as standard drug. The percentage increase in glucose uptake by yeast cells was calculated using the following formula-Increase in glucose uptake (%) = Abssample – Abscontrol X 100 Abssample Where, Abs control is the absorbance of the control reaction (containing all reagents except the test sample) and Abs sample is the absorbance of the test sample. All the experiments were carried out in triplicates.

4.4 Antimicrobial Screening of Methanolic Extract Using Agar Diffusion Method

Worldwide, infectious disease is one of main causes of death accounting for approximately one-half of all death in tropical countries. Perhaps it is not surprising to see these statistics in developing nation, but what may be remarkable is that infectious disease mortality rates are increasing in developed countries, such as the united states

Death from infectious disease rankd 5th in 1981, has become the 3rd leading leading cause of death in 1992; an increasing of 58%. It is estimated that infectious disease is the underlying cause 58% of the death occurring in the US **.**The respiratory tract infection are increased and HIV/AIDS. Other contributing factors are an increase in antibiotic resistance in nosicomial and community acquired infections. Furthermore, the most increasr are occurring in the 25-44years old age group **.**

The negative health trend call for a renewed interest in infection disease in the medical and public health communities and renewed strategies on treatment and prevention. It is this last

solution that would encompass the development of new antimicrobial .

The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility of various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the in vitro fungal and bacterial growth. This ability estimated by disc diffusion.

Some investigator use the diameter of Zone of inhibition and the minimum weight of extract inhibit the growth of microorganisms/ However, a great number of factors, the extraction methods, inoculums volume, culture medium composition, pH and incubation temperature can presence the results .

Among the above mentioned techniques the disc diffusion is a widely accepted in vitro investigation for preliminary screening of test agents which may possess antimicrobial activity. It is essentially a quantitative and qualitative test indicating the sensitivity or resistance of the microorganisms to the test materials. However, no distinction between bacteriostatic and bactericidal activity can be made by this method.

4.4.1 Principle of disc diffusion Method

The agar diffusion test, or the Kirby-Bauer disk-diffusion method, is a means of measuring the effect of an antimicrobial agent against bacteria grown in culture . The bacteria to be used are swabbed uniformly across a culture plate. A filter-paper disk, impregnated with the compound to be tested, is then placed on the surface of the agar. The compound diffuses from the filter paper into the agar. The concentration of the compound will be highest next to the disk, and will decrease as distance from the disk increases. If the compound is effective against bacteria at a certain concentration, no colonies will grow where the concentration in the agar is greater than or equal to the effective concentration. This is the zone of inhibition. Thus, the size of the zone of inhibition is a measure of the compound's effectiveness: the larger the clear area around the filter disk, the more effective the compound .

4.4.2 Materials Required

- Filter paper discs
- Autoclave
- Nutrient Agar Medium
- Laminar air flow hood

- Petri dishes

- Spirit burner

- Sterile cotton swabs

- Refrigerator

- Micropipette

- Incubator

- Inoculating loop

- Ethanol

- Sterile forceps

- Nose mask and Hand gloves

- Screw cap test tubes

4.4.3 Test Organisms

Gram Positive Bacteria

- *Staphylococcus aureus*

- *Streptococcus pyrogeny*

- *Beta Hemolyte streptococcus*

- *Bacillus cereus*

- *Bacillus subtilis*

Gram Negative Bacteria

- *Salmonella typhi*

- *E.coli*

- *Pseudomonas*

- *Shigella dysentery*

- *Salmonella paratyphi*

- *Vibriomimicus*

- *Shigella boydii*

- *Kleb siella*

Fungi

- *Candia albicans*
- *Bacillus megaterium*
- *Saccharomyces cerevisiae*
- *Aspergillus niger*

4.4.1 The Culture Medium and Its Composition

Nutrient agar was used to conduct the antimicrobial screening using the disc diffusion method. The nutrient agar was bought from the market. Nutrient agar contains the following substances:

4.4.4.1 Ingredients

- Bacto peptone - 0.5gm
- Sodium chloride - 0.5gm
- Bacto yeast extract - 1.0gm
- Bacto agar - 2.0gm
- Distilled water (Qs) - 100ml

4.4.4.2 Preparation of the Medium

The amount of nutrient agar needed was calculated and added to distilled water is an agar bottle and mixed thoroughly. It was then autoclaved to dissolve the agar and sterilize it.

Figure 4.4.1: The autoclaved machine of East West University

4.4.4.3 Sterilization Procedure

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petridishes and other glassware were sterilized by autoclaving at a temperature of $121^{\circ}C$ and a pressure of 15-lbs/sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

Figure 4.4.3: The laminar hood of East West University

4.4.4.4 Preparation of the Test Plates

The test organisms were transferred from the subculture to petridish containing the required amount of melted and sterilized agar medium as required by the size of the dish. The bacterial and fungal suspension was taken by a loop and mixed with normal saline with the help of vortex machine. Then a sterilized cotton bud was taken and dipped into the bacterial/ fungal suspension. Then the bacterial/fungal sample is applied to the petridish with the help of this cotton bud. The swabbing was done carefully so that the microorganisms would be spread out evenly on the dish.

Figure 4.4.4: The vortex machine of East West University

4.4.4.5 Preparation of Discs

Standard Discs

These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. In this investigation Ciprofloxacin (30µg/disc) standard disc was used as the reference.

Blank Discs

These were used as negative controls, which ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves. Here the negative control used was methanol.

4.4.4.6 Preparation of sample discs with test samples

Measured amount of each test sample was dissolved in specific volume of solvent to obtain the desired concentrations in an aseptic condition. For the each extract of husk, a stock solution of 10mg/ml was prepared and was used directly. Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank Petri dish under the laminar hood. Then discs were soaked with solutions of 10µl of test samples and dried.

4.4.4.7 Diffusion and Incubation

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria and fungi. The plates were then inverted and kept in an incubator at 370C for 24 hours.

***Figure 4.4.5*** Incubator for microorganisms at East West University

4.4.4.8 Determination of Antimicrobial Activity by using the Zones of Inhibition
The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs, which gives clear zone of inhibition. After incubation, the antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

RESULT

5.1 Thin Layer Chromatographic Analysis Using Different Fractions Of Methanolic Extract Of Egg Shell:

Tlcs were conducted on methanolic extract of the egg shell by using all the three types of solvent system & the best results were obtained by using the solvent system 3,which is the intremediate polar solvent system.The pictures of the plates were developed are displayed below:
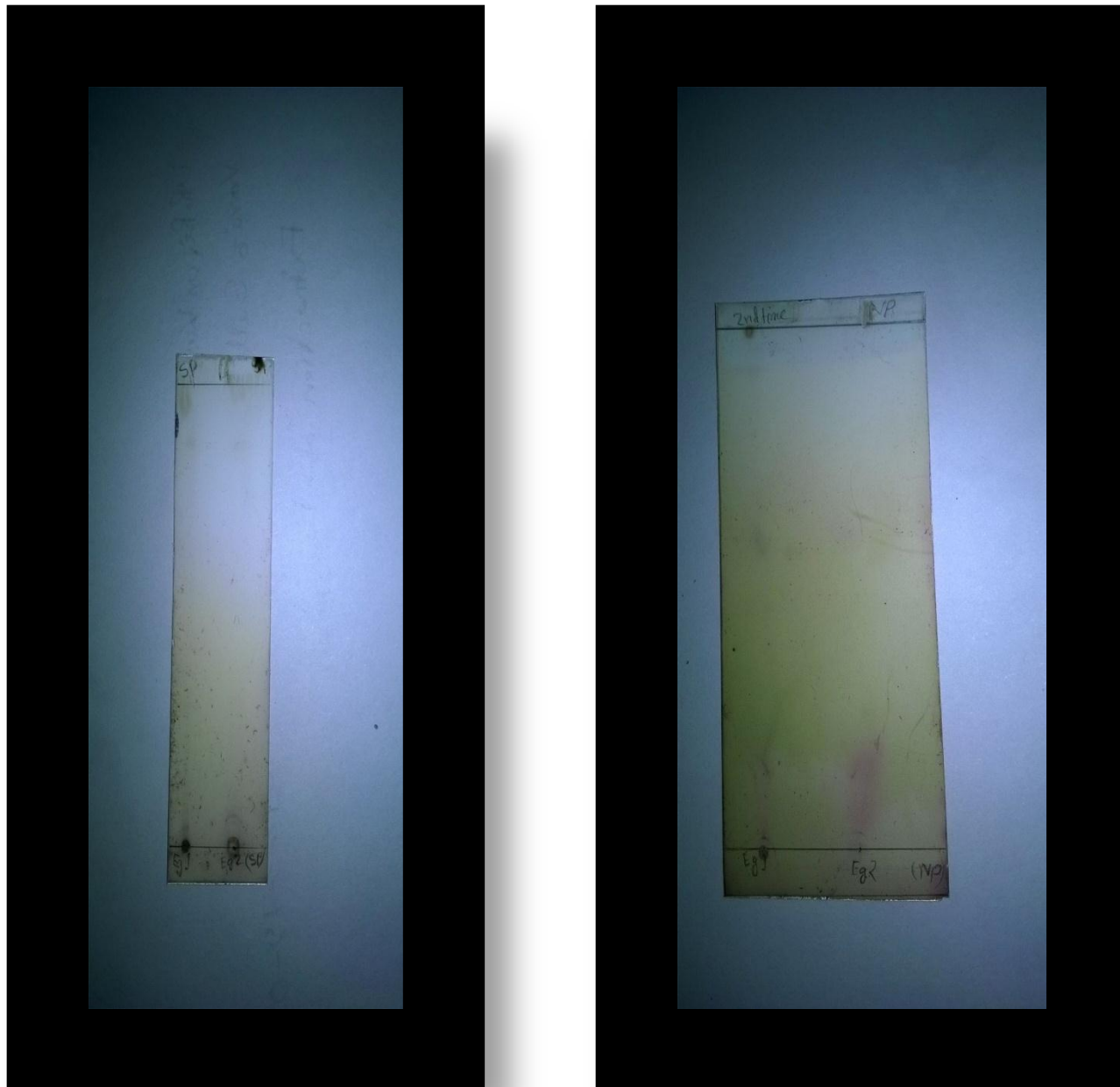
 Figure 5.1 After charring of TLC plate

Here chromatograms developed after separating the methanolic extract of egg shell using the intermediate polar basic solvent system containing chloroform, ethyl acetate, & acetic acid in the ratios of 5:4:1.The naked eye view of the TLC was not mentioned which showed no clear spot. Then the plate was observed under UV which is shown in the plate A. It showed no clear spot. Charring process done in 10% conc. Sulfuric acid on plate showed pink spot which indicates the presence of different compounds in that sample.This pink spot indicates the presence of valuable compounds that may be present in the methanolic extract of egg shell.

5.1.1 Calculation of Rf value:

The Rf value was calculated as:

Rf = Distance spot travels / Distance solvent travels.

Some Rf value (Rate of flow value) of different fraction of methanolic extract of egg shell-
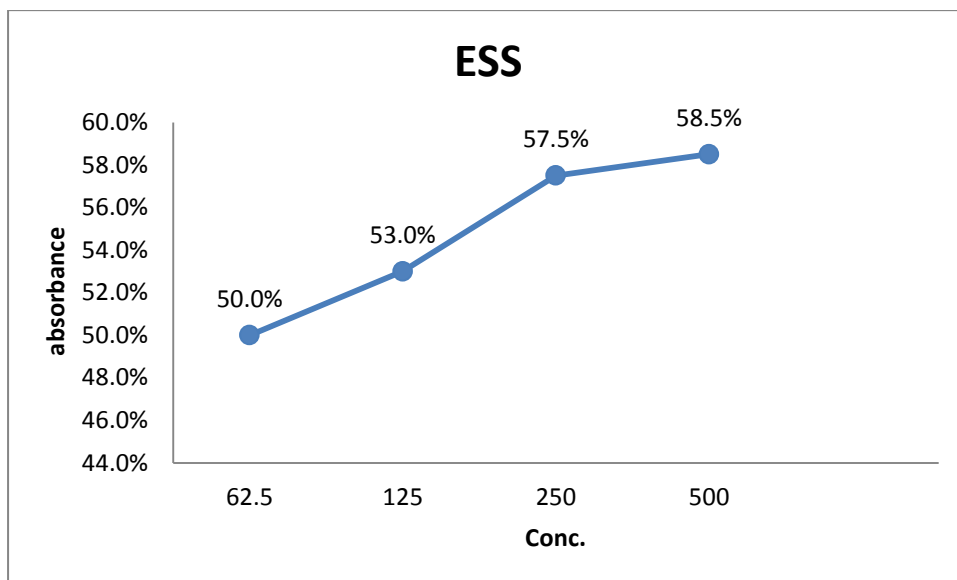
Rf value of methanol =   0.43 cm

 Rf value of Acetic =   0.54 cm

5.2 DPPH TEST

The result of DPPH test of different fraction of methanolic extract of the egg shell solid(ESS) are given in below-

Table No 5.2.1 For solid portion of methanolic extract of the *Egg Shell.*

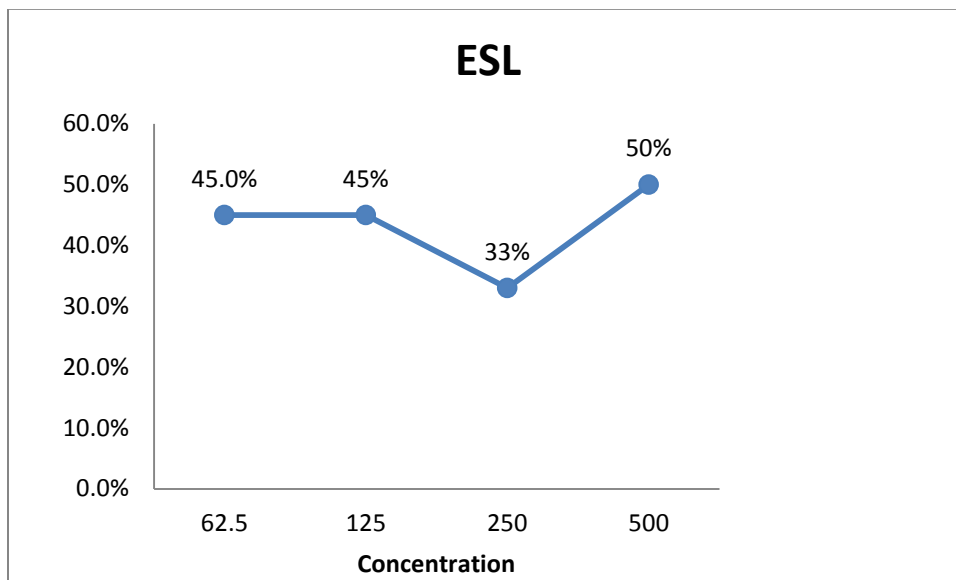| Sample Name	Concentrations µg/ml	Absorbance (517nm)	% of inhibition
	62.5	0.270	50
ESS	125	0.261	53
	250	0.262	57.5
	500	0.254	58.5

Figure5.2.1: For solid portion of methanolic extract of the *Egg Shell.*

The result of DPPH test of methanolic extract of the egg shell Liquid(ESL) are given in below-

Table 5.2.2: For liquid portion of methanolic extract of the *Egg Shell*.

Sample Name	Concentrations µg/ml	Absorbance (517nm)	% of inhibition
	62.5	0.216	45
ESL	125	0.216	45
	250	0.203	33
	500	0.218	50

Figure5.2. 2: For liquid portion of methanolic extract of the *Egg Shell*.

The result of DPPH test of different fraction of methanolic extract of the standard are given in below:

Table 5.2.3: For standard DPPH test

Sample Name	Concentrations µg/ml	Absorbance (517nm)	% of inhibition
	62.5	0.062	54
Standard	125	0.058	57
	250	0.052	61
	500	0.021	84

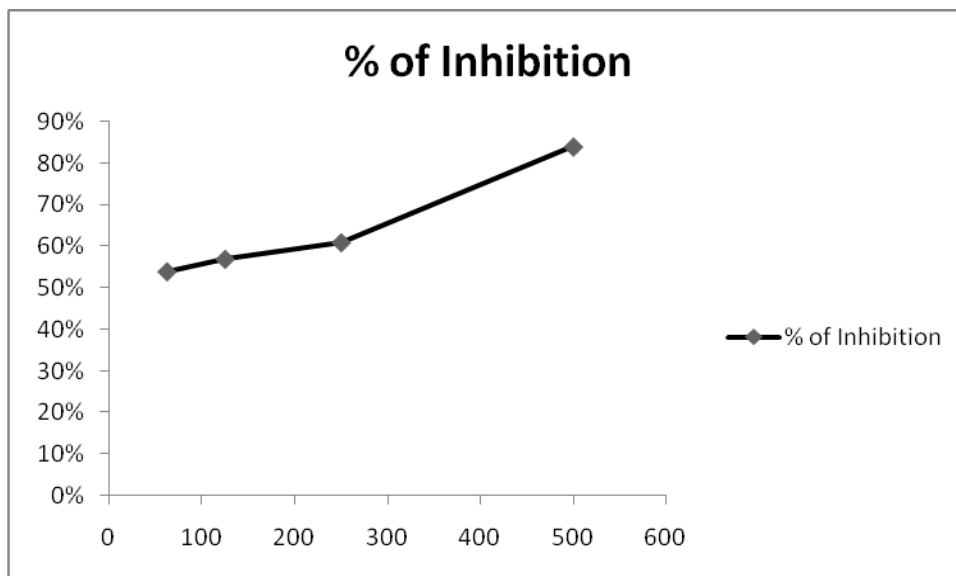
Figure 5.2.3: For standard DPPH test

5.3. IN VITRO ANTI-DIABETIC TEST

The result of anti-diabetic test of different fraction of methanolic extract of the egg shell are given in below:

Table No 5.3.1 For ESS fraction of methanolic extract of the EGG SHELL:

Sample Name	Concentrations µg/ml	Absorbance (340nm)	% of inhibition
	31.25	0.455	38
	62.5	0.479	43
ESS	125	0.490	44
	250	0.573	50
	500	0.584	51
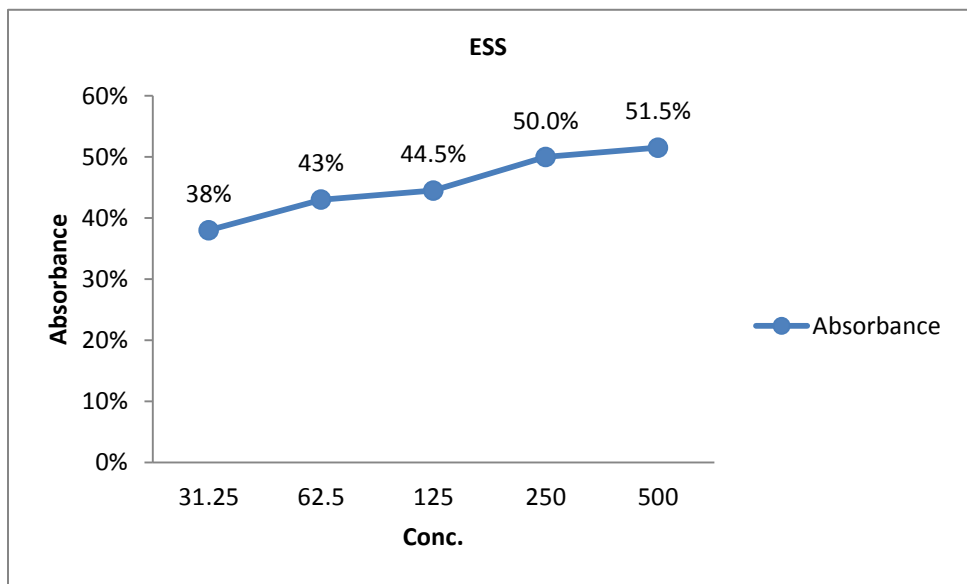
Figure5.3.1:For ESS fraction of methanolic extract of the EGG SHELL

Table No 5.3.2 For ESL fraction of methanolic extract of the EGG SHELL:

Sample Name	Concentrations µg/ml	Absorbance (340nm)	% of inhibition
	31.25	0.208	--
	62.5	0.210	--
ESL	125	0.301	6
	250	0.320	20
	500	0.421	23

Figure 5.3.3: For ESL fraction of methanolic extract of the EGG SHELL

5.4 Antibacterial activity

Figure: 5.4.1 Zone of inhibition
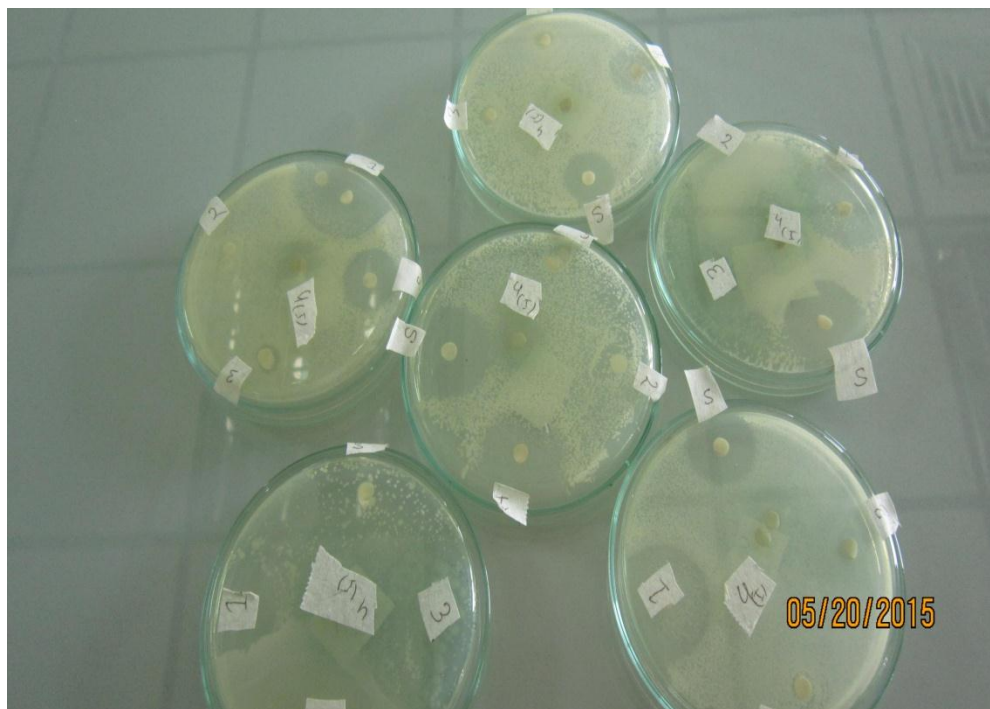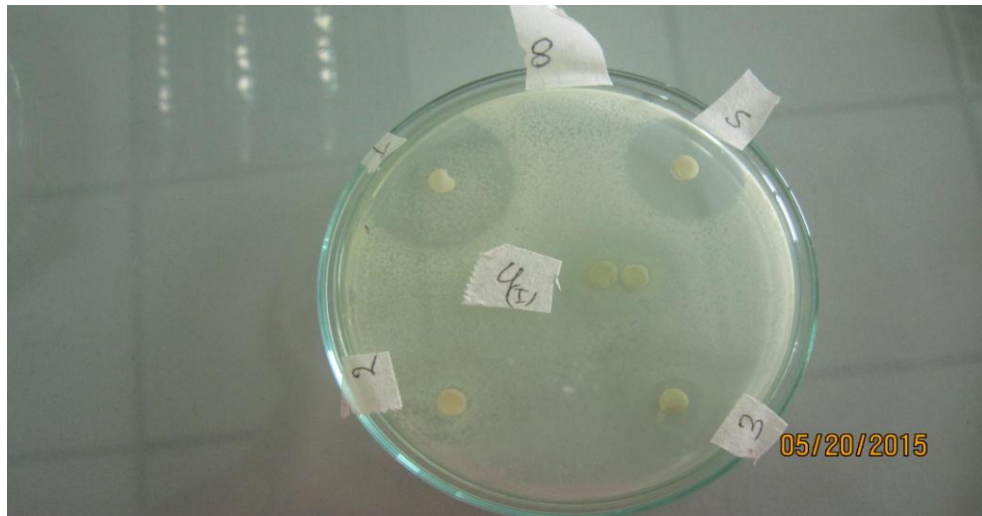
Figure: 5.4.1 Zone of inhibition

Table 5.4.1: Antibacterial activity of the methanol extract of ESS, ESL,EAS,standard and control test
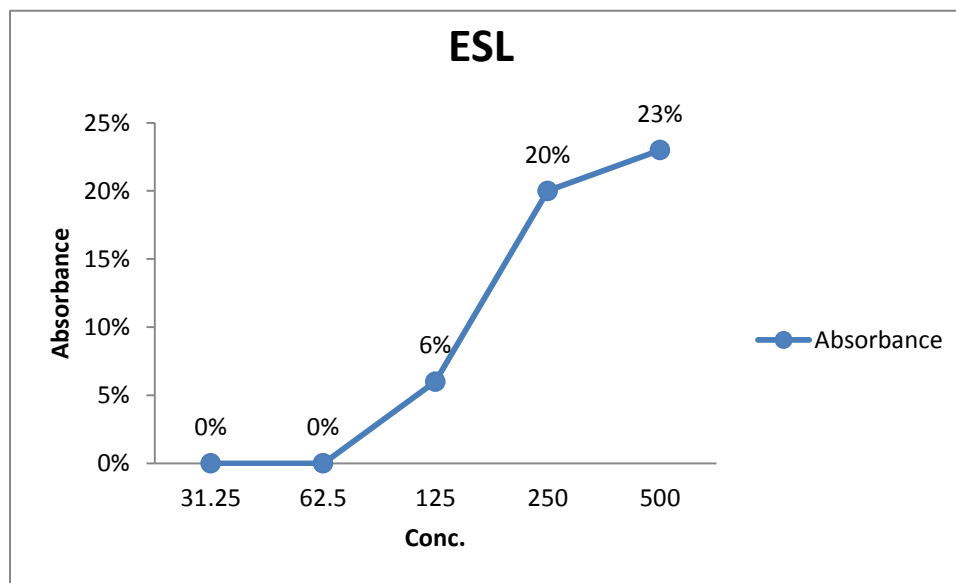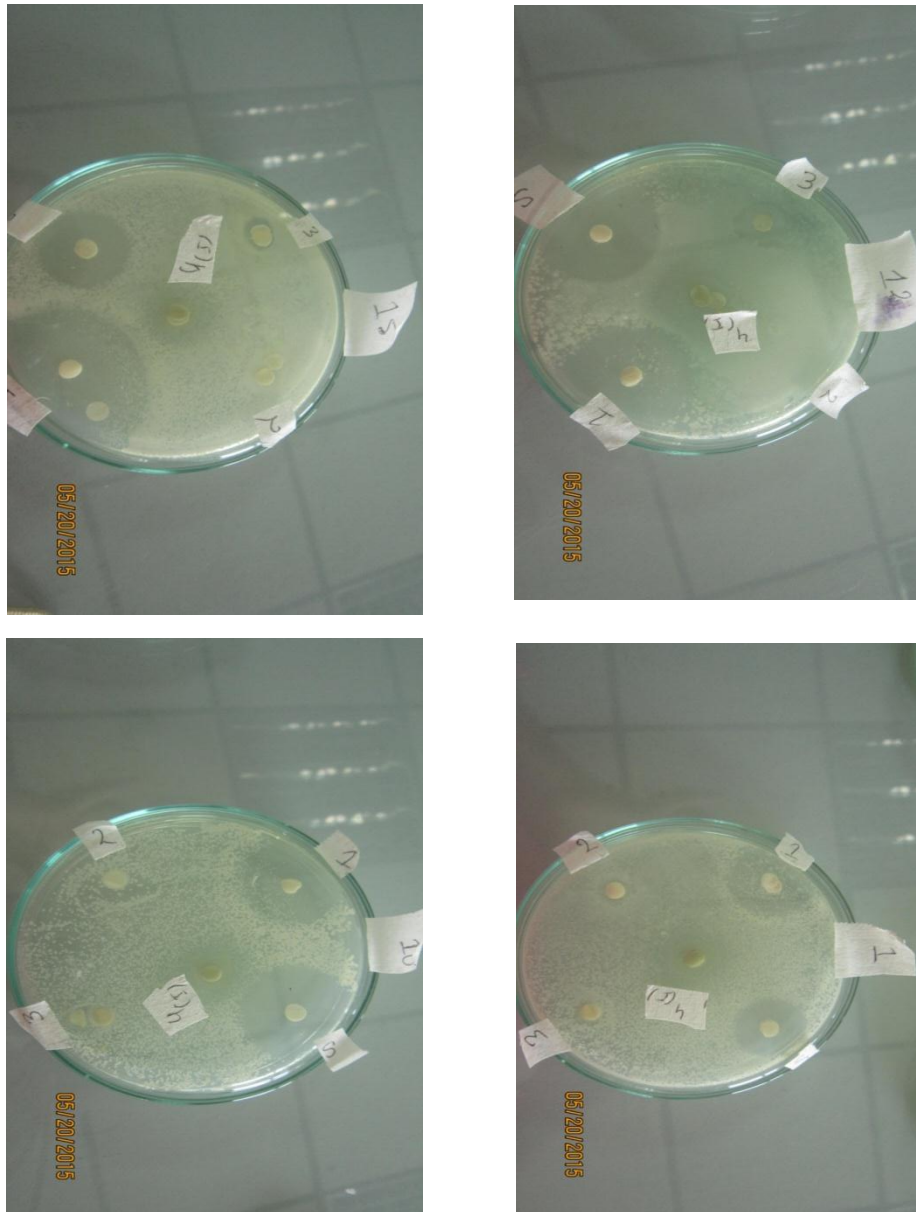
Tested bacteria	Zone of inhibition (mm)				
	EMSL	**EMSS**	**EASS**	**S (Ciprofloxacin)**	**C**
	20µg/disc	20µg/disc	20µg/disc	20µg/disc	**-**
Bacillus sereus	7.5±1	7.2±1.5	20.0±2	29.5±1.2	**-**
Bacillus subtilis	7.5±2	4.4±3.5	18.9±1.5	34.2±2	**-**
Staphylocuccos aureas	7.2±1	8.12±1.6	21.5±2.5	28.8±1.5	**-**
E.colli	7.5±1.2	7.78±2	31.25±1.6	32.4±1.0	**-**
Shigella dysentriae	6.5±1.5	6.3±1.5	30.0±1.5	31.2±1.2	**-**
Sarcina lutea	6.8±1	7.0±1.3	30.5±2.2	30.0±2	**-**
Candida albicans	6.6±1.13	11.3±4	26.0±1.2	31.2±1.0	**-**
Aspergillus Niger	6.5±1	7.3±2	30±1	33.3±1.5	**-**

Discussion

6.1 THIN LAYER CHROMATOGRAPHY

Discussion

The spot present in the plate indicates the presence of valuable compound in the methanol fraction of egg shell solid which is spotted.The Rf value of methanol = 0.43 and Rf value of acetic = 0.54. This is indicates the extensive presence of valuable compounds. From which we can have a preliminary idea of the valuable compounds that may be present in the methanolic extract of *Egg Shell*. In TLC using basic intermediate polar solvent where is spot.

6.2 DPPH TEST

Discussion

To evaluate the antioxidant activities of different fraction of methanolic extract of Egg Shell DPPH Free Radical Scavenging Assay was used.DPPH reaction has been widely used to test the ability of compounds to act as free-radical scavengers or hydrogen donors and to evaluate the antioxidant activity of foods and plant extracts. DPPH is a stable free radical that can accept an electron of hydrogen radical to become diamagnetic molecule. The reduction in DPPH radical was determined by the decrease of its absorbance at 517 nm (in methanol) induced by antioxidants (Ziying et.al.,2007).

The various fraction of methanolic extracts of the Egg Shell used in this study for evaluation of antioxidant activity, Methanol fraction of Solid of Egg Shell and Liquid of Egg Shell showed highest and lowest antioxidant activity 58.5 % and 30 % respectively.. Absorbance values and percent inhibition of each fractions were shown in table no.5.2.1.-5.2.6.

6.3 IN VITRO ANTI-DIABETIC TEST

Dicussion

In the Glucose uptake in Yeast cells method the mechanism of glucose transport across the yeast cell membrane has been receiving attention as in vitro screening method for hypoglycaemic effect of various compounds / medicinal plants. Recent studies on the transport of non metabolizable sugars and certain metabolizable glycosides suggest that sugar transport across the yeast cell membrane is mediated by stereospecific membrane carriers. It is reported that in yeast cells (Saccharomyces cerevisiae) glucose transport is extremely complex and it is generally agreed that glucose is transported in yeast is by a facilitated diffusion process. Facilitated carriers are specific carriers that transport solutes down the concentration gradient. This means that effective transport is only attained if there is removal of intracellular glucose.

In our result, Solid portion of Methanolic Egg Shell showed highest and Liquid portion of methanolic extract of the *Egg Shell* showed lowest anti-diabetic activity 51.5% and 6 % respectively.

6.4Antibacterial acivity

The antibacterial activity of the Methanol extract of *Egg Shell* were evaluated by disc diffusion method against gram positive and gram negative bacteria using ciproxacillin as standard. The Solid portion of Egg Shell, Liquid portion of Egg Shell & Acetic acic portion of Egg Shell shows varying degrees of antibacterial activities with zone of inhibition ranging from 6.5-30.0mm respectively, while the highest antibacterial activity was seen against with*, Shigella dysentriae ,   Escherichia coli.*

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CONCLUSION

The present study discusses the significance of *Egg Shell* as a valuable source for medicinally important compounds besides its seed which is a store house of , antioxidants and other nutrients..

Therefore, The present study on the different fraction of methanolic extract of the *Egg Shell* showed the potentiality of its as an antioxidant, less effect of in vitro anti-diabetic activities and. Besides, the shell may showe anti-inflammatory activity which may be induced due to its antioxidant activity.The shell showed antibacterial effect upon the pathogenic bacteria. It can call the "natural" antibacterial effect to pathogenic bacteria.

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