DETECTION OF LECTIN GENE IN *Piliostigma thonningii* AND *Albizia lebbeck* SEEDS AND THE ANTIMICROBIAL PROPERTIES OF THE PARTIALLY PURIFIED LECTIN.

BY

Oluchi Ekwutosi NWOSU, B. Sc. Biochemistry (UNIBEN) 2008
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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES,
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DECLARATION

I declare that the work in this Thesis entitled "Detection of Lectin Gene In *Piliostigma thonningii* and *Albizia lebbeck* Seeds and The Antimicrobial Properties Of The Partially Purified Lectin" has been carried out by me in the Department of Biochemistry. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other Institution.

Oluchi Ekwutosi NWOSU	-------------------------------------	-------------------------------
M.Sc/SCIE/00140/10-11	Signature	Date

CERTIFICATION

This thesis entitled, "DETECTION OF LECTIN GENE IN *Piliostigma thonningii* AND *Albizia lebbeck* SEEDS AND THE ANTIMICROBIAL PROPERTIES OF THE PARTIALLY PURIFIED LECTIN" by Oluchi Ekwutosi NWOSU meets the regulations governing the award of the Master of Science degree in Biochemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Prof. H.M Inuwa. Chairman, Supervisory Committee	------------------ (Signature)	----------------- Date
Prof. E. Onyike. Member, Supervisory Committee	----------------- (Signature)	----------------- Date
Prof. I. A. Umar Head of Department	----------------- (Signature)	------------------ Date
Prof. A. Z. Hassan Dean, School of Postgraduate Studies	----------------- (Signature)	----------------- Date

DEDICATION

This Thesis is dedicated to the Almighty God, the Most Gracious, Merciful and Faithful and to my parents Mr Chidoleum and Mrs Ngozi Nwosu.

ACKNOWLEDGEMENT

First, my thanks go to the Almighty God who gave me the strength and grace to carry out this research.

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ABSTRACT

Lectins were extracted from the seeds of *Piliostigma thonningii* and *Albizia lebbeck,* using phosphate-buffered saline (PBS) at pH 7. Agglutination reaction using chicken erythrocytes was performed to determine the presence of lectin. Purification of the extract was accomplished by ammonium sulfate precipitation and gel filtration using Sephadex G-75. The purified *P. thonningii* lectin (PTL) and *A. lebbeck* lectin (ALL), obtained when treated with denaturing and reducing conditions in the presence of sodium dodecyl sulphate (SDS) and 2-β, mercaptoethanol were electrophoresed using Poly Acrylamide Gel Electrophoresis (SDS-PAGE). The purified protein for *A. lebbeck* lectin, had a molecular weight of 21kDa as estimated from the standard protein marker while that of *P. thonningii* lectin, no distinctive band was observed. Both plant seeds crude protein and the partially purified lectin showed no antimicrobial activity against the microorganism (*Staphylococcus aureus, Escherichia coli, Salmonella typhi, Bacillus subitilis* and a fungi, *Candida albicans*) tested. The DNA of each plant was isolated using the Zymo Research Plant/Seed Kit, and the optimized Polymerase Chain Reaction used to detect the gene that codes for a galactose specific lectin protein using the primer pair LEC5 (Forward) and LEC6 (Reverse) produced a 195bp product confirming the presence of lectin gene in both plant seeds. This study has successfully confirmed the presence of lectin in both plants using the conventional hemagglutination reaction and Polymerase Chain Reaction, a method that can be applied to a variety of samples for a sensitive and specific detection of lectin in plants.

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ABBREVIATION, GLOSSARY AND SYMBOLS

PBS	=	Phosphate Buffer Saline
et al	=	And others
Rpm	=	Rotation Per minute.
%	=	Percentage
µl	=	Microlitre
mg	=	Milligram
mM	=	Millimolar
^{0}C	=	Degree Celsius
min	=	Minute
OD	=	Optical Density
Da	=	Dalton
kDa	=	kilodalton

CHAPTER ONE

INTRODUCTION

Lectins are carbohydrate-binding proteins that specifically recognize diverse sugar structures and mediate a variety of biological processes (Florin *et al*., 2011). They are proteins, other than antibodies and enzymes, that bind specifically and reversibly to carbohydrates, resulting in cell agglutination or precipitation of polysaccharides and glycoconjugates. Lectins are ubiquitous in nature, found in all kinds of organisms, from virus to humans (Sharon, 2007). Hundreds of different lectins have been isolated from plants. About 1-10% of the total soluble protein of legume seeds is composed of lectins (Savithiry *et al.*, 2013).

Generally, lectins are classified into five groups on the basis of their affinity for (i) Glucose/Mannose; (ii) Galactose and N-acetyl-D-galactosamine; (iii) N-acetylglucosamine; (iv) L-fucose, and (v) Sialic acid (Himansha and Sarathi, 2012).

Based on evolutionary and structural relatedness, Peumans and Van Damme (1998), placed the lectins into seven families: legume lectins, Chitin binding lectins, Type-2-ribosome-inactivating proteins (RIPs), monocot mannose binding lectins, *Amaranthins*, *Cucurbittaceae* phloem lectins and jacalin related lectins. Among all these the legume lectins are extensively studied and are present in several hundreds of species of legumes. However, almost all well-characterized *Leguminosae* lectins are from the *Papilinoidae* subfamily, and only few lectins from the *Caesalpiniacea* species have been reported, such as *Bauhinia purpurea*, *Griffonia simplicifolia* and *Bauhinia variegata* (Pinto *et al.*, 2008).

Piliostigma thonningii is a leguminous plant belonging to the sub family *Caesalpiniacea*, a family that comprises of trees, shrubs or very rarely scramblers. Different parts of *P.*

thoningii have also been described as useful medicinal plant. Its root and twig have been used for the treatment of dysentery, fever, respiratory ailments, snake bites, hookworm and skin diseases (Jimoh and Oladiji, 2005).

Albizia lebbeck benth, known as *Shirisha* in Hindi is an important medicinal plant belonging to the family *Fabaceae* (*Leguminosae*) and a member of subfamily *Mimosaceae*. The plant is useful in many disease conditions and is known for its anti-inflammatory, anti-histaminic, anti-anaphylactic, anti-asthmatic and anti-microbial activities (Shyamlal *et al.*, 2011).

Lectins may be toxic, inflammatory, resistant to cooking or digestive enzymes, and are found in much of our foods. Even though, lectins are associated with toxicity, their importance in terms of cancer therapeutics, immunology, and antibacterial property cannot be neglected. Scientific research has shown that few of the peculiar characteristics of plants (anti-bacterial properties, cytotoxicity) are because of the presence of lectins and the understanding of these characteristics at molecular level could only be gained thorough investigation of their associated lectins (Himansha and Sarathi, 2012).

This Research was designed to confirm the presence of lectin genes in *Piliostigma thonningii* and *Albizia lebbeck* seeds, using conventional agglutination reactions, polymerase chain reaction and also to establish its antimicrobial activity.

1.1 STATEMENT OF RESEARCH PROBLEM

Commercial availability of a protein at a reasonable price is a pre requisite for its intensive use as a research tool. Although the preparation of plant lectins is in principle, relatively easy because powerful affinity chromatography can be included in the purification scheme, many interesting lectin cannot be prepared on a large enough scale for practical reasons (Peumans and Van Damme, 1998). In some cases the starting material is not available in sufficient quantities. For instance, the preparation of large quantities of the orchid lectin (which exhibits a very interesting specificity and have unique biological activities) is virtually excluded because these species are protected. In other cases, the lectin concentration in the plant materials is so low that the cost for the preparation of the purified proteins impedes their commercialization. The later holds true, for instance, for the lectin from onions or leek (Peumans and Van Damme, 1998). Even when the lectin concentration in a given plant tissue is reasonably high, the preparation of the pure protein may be very cumbersome because of the presence of large quantities of polysaccharides, phenolic compounds or interfering substances. The complex and costly procedure required for purification of these lectins makes them unattractive for large scale isolation. For instance, problems caused by high level of pectic substance and anthocyanin in elderberry fruits makes a cost effective purification of the fruit specific lectin virtually impossible (Peumans and Van Damme, 1998).

Many food plants (especially legumes and cereals) contains lectins which are believed or suspected to have negative effect on food quality and safety.

Only few lectins from the *Caesalpiniacea* species have been reported, such as *Bauhinia purpurea* (Irimura and Osawa 1972), *Griffonia simplicifolia* and *Bauhinia variegata* (Pinto *et al.,* 2008).

Several plant lectins cannot be classified because of lack of sequence information. Novel lectin families will not be discovered if more sequence data is not made available.

1.2 JUSTIFICATION FOR THE STUDY

Lectins binds glycoconjugates regardless of their origin, hence an important tool in a wide variety of studies, including cell-cell recognition and cell activation (Debray *et al.,* 2009; North *et al.,* 2012). These potential uses have motivated the continued search for new lectins and for seeds to be prioritized for screening.

Molecular cloning and analysis of plant lectin genes as well as the expression of lectins in heterologous systems are new developments offering interesting perspectives not only for a more intensive use of lectins but also for a novel application in other fields.

1.3 AIMS AND OBJECTIVES OF THE STUDY

1.3.1 Aim.

The aim of this study is to purify and to detect lectin gene in the isolated Deoxyribonucleic acid (DNA) from the seeds of *Albizia lebbeck* and *Piliostigma thonningii* using polymerase chain reaction method.

1.3.2 Specific Objectives

Specific Objectives include:

1. To determine the presence of lectin in the seeds of *Albizia lebbeck* and *Piliostigma thonningii* plants.
2. To isolate and partially purify lectin from the seeds of *Albizia lebbeck* and *Piliostigma thonningii* plant.
3. To determine out antimicrobial activity of the partially purified fraction from *Albizia lebbeck* and *Piliostigma thonningii.*
4. To determine the molecular weight of the partially purified lectin from *Albizia lebbeck* and *Piliostigma thonningii* using SDS PAGE.
5. To isolate the DNA from *Albizia lebbeck* and *Piliostigma thonningii* and amplify lectin.gene through the polymerase chain reaction.

CHAPTER TWO

LITERATURE REVIEW

2.1 Lectins

The term “hemagglutinins” or “phytoagglutinins” came into existence with the discovery of proteins possessing the ability to agglutinate erythrocytes. The first hemagglutinin capable of showing this property was reported in the castor bean extract (Peumans & Van Damme, 1998). In 1940s, it was found that some of the hemagglutinins selectively agglutinated blood cells of a particular human blood group, therefore the novel term “lectin” was proposed by Boyd and Sharpleigh (1954) for the class of carbohydrate-binding proteins, which selectively agglutinate erythrocytes.

Lectins are carbohydrate-binding proteins that specifically recognize diverse sugar structures and mediate a variety of biological processes (Florin *et al.*, 2011).They are proteins, other than antibodies and enzymes, that bind specifically and reversibly to carbohydrates, resulting in cell agglutination or precipitation of polysaccharides and glycoconjugates. Lectins are ubiquitous in nature, found in all kinds of organisms, from virus to humans (Sharon, 2007). Hundreds of different lectins have been isolated from plants. About 1-10% of the total soluble protein of legume seeds is composed of lectins (Savithiry *et al.*, 2013). They are the natural partners of the various oligosaccharides that nature uses to "tag" cells via glycoproteins and glycolipids. Lectins play a role in important processes like infection, host defence, fertilization, cancer, protein transport and embryogenesis (Teixeira *et al.*, 2012).They are also important as tools in biology, histology and medicine, e.g., as an aid in the purification of glycoproteins or the determination of blood groups (Van Damme *et al.*, 1998). Lectin-carbohydrate recognition is one of the three fundamental biological recognition mechanisms, the two others being protein-nucleic acid recognition and protein-protein recognition.

2.1.1 Classifications of Lectins

Based on the amino acid sequences of available lectins, it is deduced that the carbohydrate binding property of most lectins resides in a polypeptide sequence, which is termed as 'Carbohydrate-recognition domain' (Drikamer, 1988). Generally, lectins are classified into five groups on the basis of their affinity for (i) Glucose/Mannose; (ii) Galactose and N-acetyl-Dgalactosamine; (iii) N-acetylglucosamine; (iv) L-fucose, and (v) Sialic acid (Himansha & Sarathi, 2012).

Based on evolutionary and structural relatedness, Peumans and Van Damme (1998) placed the lectins in seven families: legume lectins, Chitin binding lectins, Type-2-ribosome-inactivating proteins (RIPs), monocot mannose binding lectins, *Amaranthins*, *Cucurbittaceae* phloem lectins and jacalin related lectins. Among all these the legume lectins are extensively studied and are present in several hundreds of species of legumes.

2.1.2 Plant Lectins

Plant lectins are usually considered as a very heterogeneous group of proteins because comparative biochemical studies clearly indicate that they differ from each other with respect to their biochemical/physicochemical properties, molecular structure, carbohydrate-binding specificity and biological activities (Van Damme *et al*, 2008).

Based on the amino acid sequence, plant lectins are sub-divided into 3 different types. These include:

a) Legume lectins,

b) Monocot mannose-binding lectins,

c) Chitin-binding lectins,

2.1.2.1 Legume Lectin

Of the plant lectins, the legume lectins as a group have been the most extensively studied (Van Damme *et al.,* 2008). The three-dimensional structure of ConA was the first to be elucidated among legume lectins (Edelman *et al.,* 1972). Many other lectins have since been purified and their structures elucidated, and some have been co-crystallized with their ligands such as the *Lens culinaris* seed lectin monomer. When crystallized, legume lectins generally consist of two or four 25–30 kDa subunits, which are dome-shaped and consist largely of antiparallel sheets. Overall, the legume lectins (L-type) whose structures are known to exhibit a similar protein-folding pattern, thus exhibiting three-dimensional structural conservation. In contrast to the generally conserved legume lectin fold structures, the major cereal lectins, a less extensively characterized family of proteins than the legume lectins (Sharon, 1993; Van Damme *et al.,* 2008), are homodimers comprised of two subunits of 18 kDa.

The first plant lectin to be purified and crystallized was Concanavalin A (Sumner and Howell, 1936) and its primary and three-dimensional structure was determined (Edelman *et al.,* 1972; Hardman and Ainsworth, 1972). The first plant lectin gene that was sequenced was soyabean seed lectin (Vodkin *et al.*, 1983).

Legume lectins are commonly glycosylated and composed of two or four protomers held together by non-covalent bonds, so that the functional lectin molecule has multiple carbohydrate binding sites. They also contain Mn^{2+} and Ca^{2+} ions associated with a series of highly conserved amino acids, which participate in carbohydrate binding. Various legume lectins may bind galactose, N-acetyl D-galactosamine, mannose, glucose, N-acetyl D-glucosamine, fucose, N-acetyl neuraminic acid, or more complex carbohydrates (Loris *et al.*, 1994).

2.1.2.2 Chitin binding lectins

These proteins consist of one hevein domain. Chitin binding lectins are found in plant families like *Gramineae*, *Solanaceae, Phytolaccaceae, Urticacea*, *Viscaceae* and *Papavaracaceae* (Raikhel *et al.*, 1993; Peumans & Van Damme, 1996). They occur mainly in seeds and vegetative tissues. They belong to different classes like merolectins, hololectins and chimerolectins. The examples of chitin binding lectins without hevein domain are chitin binding legume lectins and *Cucurbitaceae* phloem lectins (Van Damme *et al.*, 1998).

Chitin binding lectins contains hevein domains; the term “hevein‟ refers to a chitin binding polypetide of latex of *Hevea brasiliensis*. They bind N-acetyl D-glucosoamine oligomers and polymers of N-acetyl D-glucosamine. chimeric lectin is of two types:

Class I chitinases

It has a N-terminal chitin binding domain linked to active chitinase domain (Collinge *et al.*, 1993; Beintema, 1994). They help in plant’s defence against fungi.

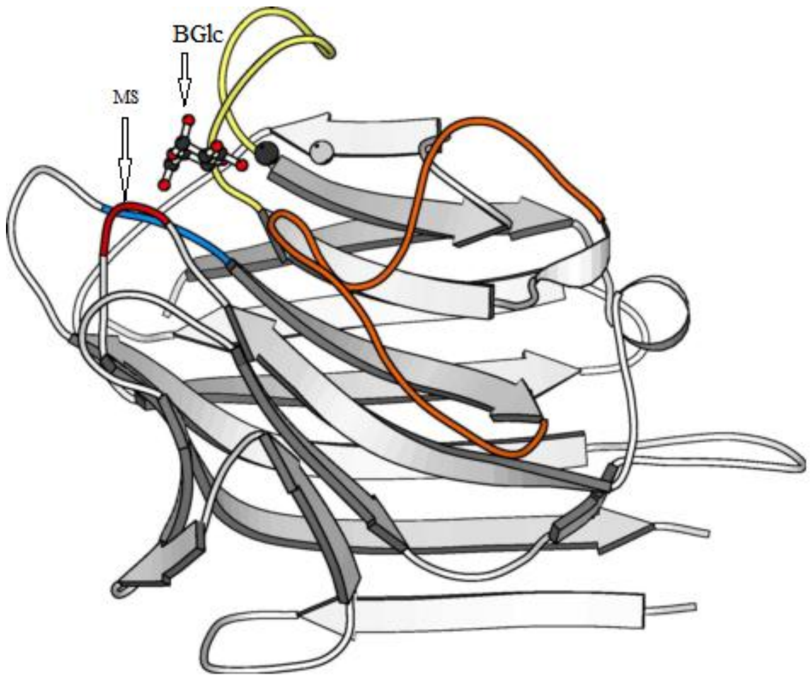


Figure 2.1: A typical legume lectin monomer (lentil lectin), PDB entry 1LES, (Loris *et al.*, 1994). Strands are shown as arrows. The conserved monosaccharide binding site is located on the top of the monomer, in a shallow depression. The bound Glucose molecule (BGlc) is shown as a ball-and-stick model. The four sugar binding loops are shown in different colors (Ala-Asp cis-peptide bond in blue; metal binding loop in yellow; Ω -loop in orange; monosaccharide (MS) specificity loop in red). The metals are shown as gray spheres (Ca^{2+} dark gray; Mn^{2+} light gray).

Class II chitinases

It consists of dimeric *Solanaceae* lectins. Its proteins are built up of chimeric polypeptides consisting of N-terminal chitin binding domain with three hevein repeats (Kieliszewski *et al.*, 1994; Allen et al., 1996). Examples are *Lycopersicon esculentum* agglutinin and *Solanum tuberosum* agglutinin.

2.1.2.3 *Monocot mannose-binding lectins*

This lectin belongs to the sub-group of monocots. They are mannose-specific lectins and are present in monocot families like *Alliaceae*, *Amaryllidaceae*, *Araceae*, *Bromeliaceae*, *Liliaceae*, and *Orchidaceae* (Van Damme *et al.*, 1998). They occur mainly in leaves, roots, flowers, ovaries, bulbs, tubers, rhizomes, roots, nectar (Peumans *et al.*, 1997).

The first lectin to be crystallized and analyzed by X-Ray diffraction (Hester *et al.*, 1995) was *Galanthus nivalis* agglutinin (GNA) and its 3D structure was determined. It has a β-prism II fold structure.

2.2 Site of Synthesis of Legume Lectin

Legume lectins accumulate in vacuoles and protein bodies. They are synthesized on the rough endoplasmic reticulum (RER) as preproteins or pre-proproteins. The precursor molecules are further processed by proteolytic cleavage of the polypeptides (pro-proteins to proteins) or trimming of sugar residues from the oligosaccharide side-chains of glycoproteins at the Golgi system (Figure 2.2). The mature lectin molecule is then transported via the endomembrane system to the site of accumulation (Chrispeels, 1984).

Lectins were first characterized as sugar-binding proteins from seeds where they function as storage proteins, and additionally may display anti-pathogenesis and anti-predation functions against microbes, insects, and herbivores (Brewin and Kardailsky, 1997). Lectin levels rapidly decrease in seed cotyledon tissue shortly after germination with the same kinetics as other seed storage proteins because they are catabolized for energy or for amino acid building blocks (Pusztai, 1991). Most plant lectins, and all known secreted ones, are synthesized on membrane-bound polyribosomes as propeptides with a signal sequence. Before introduction into the lumen of the rough endoplasmic reticulum and sequestration within the Golgi apparatus, several processing steps occur, including cleavage of the signal peptide and addition of oligosaccharides to the polypeptide chain at specific asparagine residues. Glycosylation is thought to mediate the targeting of the lectins to their final destination (Pusztai, 1991). In addition, given the presence of the calnexins and calreticulins in plants, previously described as being involved in protein folding checkpoint processes, and the extensive hydrophobic cores of lectin proteins, glycosylation events may also facilitate proper lectin folding prior to export (Banerjee *et al.*, 2007). These steps are followed by accumulation in protein storage bodies or signal sequence-mediated protein localization (Chrispeels, 1984; Lord, 1985; Vitale and Bollini, 1995). Lectins stored within vacuoles or protein bodies are likely to function in defense because when an invading microbe or insect disrupts the plant's cellular contents, the stored lectin, which may agglutinate or sicken the pathogen or predator, is released (Chrispeels and Raikhel, 1991). Legume lectins are also immunolocalized to roots and root hair tips (Díaz *et al.*, 1995; Van Rhijn *et al.*, 1998) and secreted into the extracellular environment (Wen *et al.*, 2007), most likely as a consequence of the default secretion pathway that is mediated by the fusion of Golgi vesicles to the plasma membrane. However, it has yet to be elucidated if the secreted plant lectins are proteolytically cleaved from the membrane or are bound to other proteins or to the membrane itself, because galectins are released from outer membranes by enzymatic digestion of surface carbohydrates (Garner and Baum, 2008), it is possible that soluble plant lectins are also linked to a region near the plasma membrane through interactions with surface glycoproteins. Secretion of plant lectins into the medium may enable a role in defense or symbiosis via bacterial or fungal binding and subsequent agglutination.

Cereal lectins such as wheat germ agglutinin (WGA) are also synthesized as propeptides and secreted (Vitale and Bollini, 1995). Additionally, WGA and its homologs have been immunolocalized to the coleoptiles of embryos and to the epidermal cells of aerial and subterranean organs of wheat, rice, and barley, serving in these locations as potential mediators of plant defense (Mishkind *et al.,* 1982, 1983). The advent of genomics and the application of mRNA expression profiling have allowed the discovery of large numbers of genes with lectin or lectin-like domains in legumes and in plants in general, shedding new light on the lesser-studied endogenous roles of lectins in plants. In contrast with earlier findings showing that lectins are expressed primarily in seeds, microarray data demonstrate that putative lectin genes are expressed in a multitude of tissues showing a mixture of unique and overlapping expression patterns (Schmid *et al.,* 2005; Benedito *et al.,* 2008; Sato *et al.,* 2008). Basal lectin expression levels vary by organ structure, and can change in response to microorganisms or to other environmental stimuli.

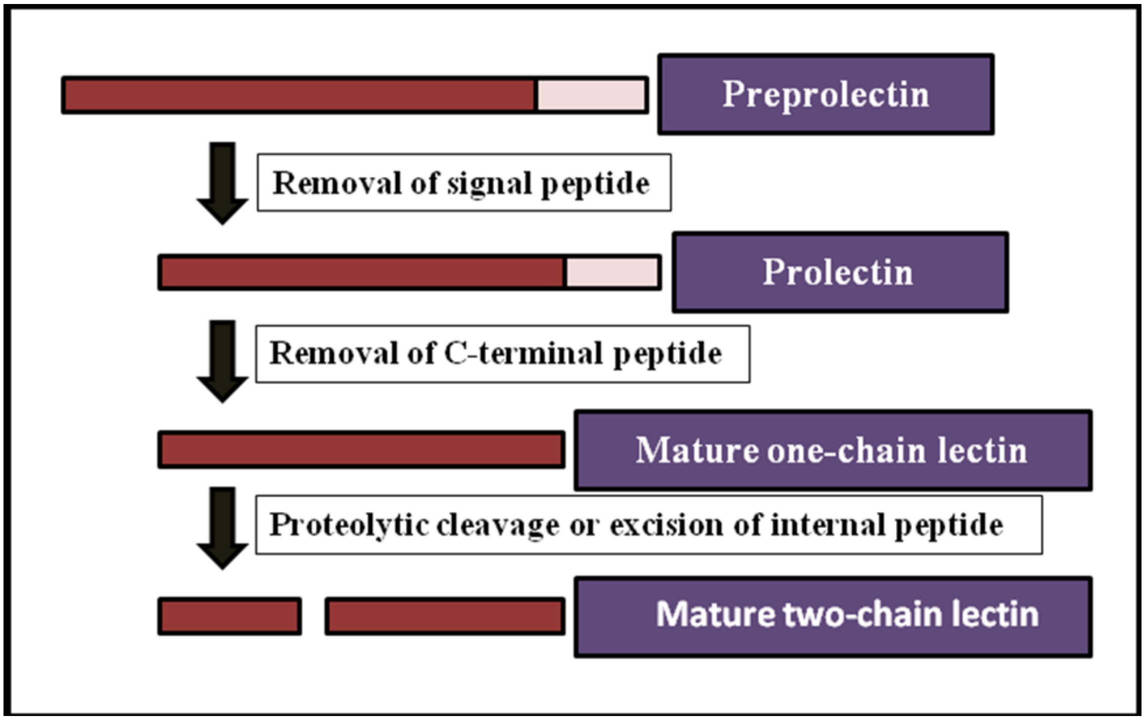


Figure 2.2: Schematic representation of the processing of one-chain and two- chain legume lectin (Mandal *et al.*, 1994).

2. 3 Biological Role of Lectins

In spite of being the most thoroughly studied lectins, the functions of the plant lectins remain enigmatic. Proposed functions for plant lectins include a storage or transport role for carbohydrates in seed, binding of nitrogen fixing bacteria to root hairs and inhibition of fungal growth or insect feeding. All cells are coated with sugars and many also express surface lectins (Barira and Asad, 2012). Cell surface sugars and lectins on cell surfaces are believed to function as recognition determinants between cells, either homotypic or heterotypic. For instance, the interaction between lectins on bacterial surfaces and sugars on eukaryotic cells play a crucial role in the infection process (Barira and Asad, 2012).

Lectins play a major role in nitrogen fixation in leguminous and non-leguminous plants. It has been suggested that root lectin recognized by bacterial receptor molecules is an important determinant of host plant specificity in Rhizobium legume symbiosis (Bohlool and Schmidt, 1974).

In addition to these roles, evidences exist for their involvement in embryogenesis, development and reproduction. Two lectin genes MsLEC1 and MsLEC2 from alfalfa cloned in antisense orientation and expressed in alfalfa, lead to severe embryogenic, developmental and reproductive abnormalities (Brill *et al.,* 2001).

Lectins serve as means of attachment of different kinds of cells as well as of viruses to other cells via the surface carbohydrates of the cells to be attached (Kapoor *et al.,* 2014). In some cases, cell surface lectins bind to particular glycoproteins, whereas in other cases the carbohydrates of cell surface glycoproteins or glycolipids serve as sites of attachment for biologically active molecules that have specificity towards carbohydrate, for example, microorganisms, various plant toxins, galactic and similar compounds as shown in Figure 2.3.

Plant lectins also function as plant defence proteins. Plant lectins affect various biological parameters of insects which include larval weight decrease, mortality, feeding inhibition, delay in total developmental duration, adult emergence and fecundity on the first and second generation (Habibi *et al.,* 1993; Powell *et al.,* 1993). Plant lectins can either directly or indirectly cause profound morphological and physiological modifications in the insect intestine. In insects, lectins bind to the midgut epithelium causing disruption of the epithelial cells including elongation of the striated border microvilli, swelling of the epithelial cells into the lumen of the gut lead to complete closure of the lumen and impaired nutrient assimilation by cells, allowing absorption of potentially harmful substances from intestine into circulatory system, fat bodies, ovarioles and throughout the haemolymph (Habibi *et al.,* 2000; Fitches *et al.,* 2001).

Beside their insecticidal properties, lectins have also been found to be effective against transmission of plant viruses. Certain plant viruses are transmitted by insects of the Hemiptera order (sap-sucking insects) including aphids, whiteflies, leafhoppers, planthoppers, and thrips. The mode of transmission can either be by moving from the alimentary canal of the vector insect onto its hemocoel and through the salivary secretory system into the plant host during insect feeding or by associating with the cuticular lining of the insect mouthparts or foregut and directly releasing as digestive secretions onto the plant when insect begins to feed (Gray and Gildow, 2003; Hogenhout *et al.*, 2008).

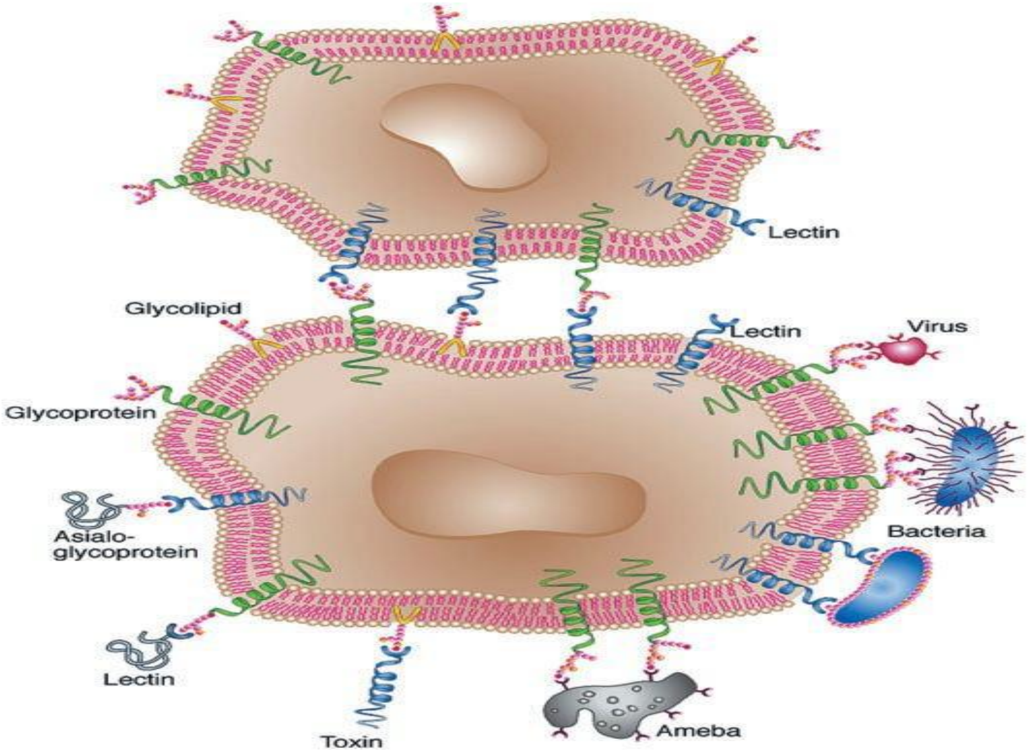


Figure 2.3: Specificity of cell surface carbohydrates towards various biomolecules. (Kapoor *et al.*, 2014)

Lectins are able to recognize and bind to the viral glycoproteins thereby decreasing the binding of virus with the receptor and subsequently avoid the transport of virus from gut to hemocoel of insect vector (Desoignies, 2008), finally suspending the virus transmission.

Binding of plant lectins to bacterial cell wall peptidoglycans indicate that lectins strongly interact with muramic acid, N-acetyl muramic acid and muramyl dipeptide and play direct role in plants defense against bacteria. The definitive proof for antifungal activity of plant lectins was demonstrated by a purified lectin from stinging nettle (*Urtica dioica*) which inhibited the growth of *Botrytis cinerea*, *Trichoderma hamatum* and *Phycomyces blakeslecanus*. Similarly, Hevein, a lectin from latex of rubber tree and a lectin from seeds of *Amaranthus caudatus* have antifungal activities (Lam and Ng., 2011).

Feeding trials with purified lectins from snowdrop (*Galanthus nivalis*) and garlic (*Allium sativum*) indicated that they are moderately active against chewing insects, such as cowpea weevil and tobacco hornworm (Spodoptera *litoralis*) but are not toxic to mammals (Pusztai *et al.*, 1992). Snowdrop lectin is the first to be effective on a sap sucking insect, rice brown plant hopper (Powell *et al.*, 1993). Similarly, lectin from *Canavalia ensiformis* (Con A) was a potent toxin of the pea aphid *Acyrthosiphon pisum* with no toxicity on mammals. *Galanthus nivalis* agglutinin (GNA) caused 79% mortality to first-instar nymphs of rice brown plant hopper (*Nilaparvata lugens*) and 89% mortality to rice green plant hopper (*Nephottetix cinciteps*) whereas most other lectins had little or no effect.

2.4 Lectins : Toxins or Therapeutics

Toxic relevance of lectins is known since ages. Some lectins are resistant to the gut enzymes and do not break easily, they might bind to the wall of the gut and cause damage to the gut lining. This could be related to diseases such as colitis, Crohn's disease, and Coeliac-Sprue. Years of research has shown that after the interaction of lectin with the intestine, it is edocyted and cause many disturbances in the systemic levels (Himansha and Sarathi., 2012). Few similar experiments were performed by De oliveria and his colleagues, they concluded that when lectin derived from pure Phaseolus vulgaris were fed to rats, enlargement of the intestine, liver, and pancreas was observed. Apparently, this enlargement in pancreas may be responsible for the observed decrease in the insulin levels of the rats (Himansha and Sarathi., 2012). It was also observed that the kidney bean lectin fed rats had thymus atrophy and it is speculated that this atrophy developed may be because of the unusual proliferation of bacteria in the gut, as the immunological system of the rats may have been depressed due to toxic effects of lectins. The ingestion of kidney bean lectin also disturbs the intermediary metabolism, leading to loss of weight, inadequate development and eventually death of the experimented rats (Himansha and Sarathi, 2012). Other diseases associated with lectins are insulin dependent diabetes, rheumatoid arthritis, IgA nephropathy and peptic ulcers. Lectins sensitivity could occur due to the failure of a certain type of barrier protection in the body, namely Secretory Immunoglobulin A (sIgA) barrier protection. It could be argued that lectins are specific to certain carbohydrates and when they attach to their specific carbohydrate substrate, they may damage the cell membrane and may damage the cell (Himansha and Sarathi., 2012).

Although, lectins possess several toxic elements, are used in various biological fields. Their contribution in Cell identification and separation, detection, isolation, and structural studies of glycoproteins, investigation of carbohydrates on cells and subcellular organelles (Himansha and Sarathi, 2012), mapping of neuronal pathways, mitogenic stimulation of lymphocytes, purging of bone marrow for transplantation and studies of glycoprotein biosynthesis is remarkable (Wang *et al.*, 2006). Certain lectins are also used as carriers for the delivery of chemotherapeutic agents; they are also used in investigating cell surface receptors in various bacteria, protozoa and fungi. Lectins can also be used in determining bacterial cell wall components and bacteriophage receptors. Lectins are generally monoclonal proteins and they possess a spectrum of specificities and molecular weights, due to this, they are classified as substantial tools for diagnostic microbiology application (Kapoor *et al*., 2014). One of the advantages of applying lectins in clinical microbiology is that cellular or surface receptor sites can be partially analyzed by hapten inhibition studies (Kapoor *et al*., 2014). Unlike the production of antisera, which requires pre-treatment of microorganisms for antigen preparation followed by injection of the microorganism and glyconjugate into animals, such as rabbits leading to the absorption of antisera to eliminate nonspecific antibody reactions, lectins are simple to use, when they are conjugated to a histochemical label such as fluorescein, peroxidase, or colloidal gold, lectins may be used as histochemical probes to identify and localize specific carbohydrate residues in microorganisms by light or electron microscopy, as well as by blotting methods (Kapoor *et al*., 2014). The cell binding property of lectins elicit a wide range of biological phenomena for instance, lectins have been used to fractionate animal cells, including B and T lymphocytes and also illustrates changes in cell surface architecture following virus infection or parasite infection (Himansha and Sarathi., 2012). They are very important and versatile tools and are applied as probes for fluorescence and electron microscopy as well as in gel diffusion assays. Immobilized lectins are used for affinity chromatography during the separation of glycoproteins as they have advantage over other purification techniques because elution can be pursued with a relatively inexpensive monosaccharide and the glycoprotein, which is to be purified, is not subjected to denaturation (Himansha and Sarathi., 2012).

Over the past few years, Lectins have been found to have anticancer properties. Several researches have shown the use of Lectins to inhibit tumor growth, especially by causing cytotoxicity, apoptosis and, down-regulation of telomerase activity and inhibition of angiogenesis. In addition, lectins have also been found to sequester the pool of available polyamines in the body; thereby help in thwarting cancer cell growth (De Mejia and Prisecaru, 2005).

Some lectins are potent toxins, but due to this ability they could be used as potential therapeutic agents, for instance, lectins such as 'Ricin' and 'Abrin' have been coupled to specific monoclonal antibodies and are used in cancer therapy. Ricin could be used for developing specifically cytotoxic chemotherapeutic agents by linking them to cell type-specific monoclonal antibodies. Cancer is a deadly disease, where the aberrant behavior of a single cell type is difficult to treat by chemotherapy (Himansha and Sarathi, 2012). It is important in cancer therapy that the treatment targets only the affected cells, leaving the normal cells undisturbed, which is quite difficult, especially in chemotherapy. A promising approach is to incorporate a hybrid reagent that has selectivity towards target cells with potential cytotoxicity. Immunotoxins (ITs) conjugates in which cell reactive monoclonal antibodies are chemically joined to potent toxins are most popular manifestation of such hybrids. Target cell specificity is conferred by a monoclonal activity that has been raised against the tumor cell-specific surface antigen (Himansha and Sarathi, 2012).

2.5 Lectin Agglutination Reaction.

Lectins are found in cells, membranes, and secretomes of organisms from all kingdoms of life, and tolerate a degree of binding-pocket sequence variation for ligand specificity rivaling that of immunoglobins (McMahon *et al.*, 2005). Carbohydrates are a far more diverse group of molecules than proteins, owing to the variability in their constituent saccharide monomers coat i.e. cell wall (plant) or glycocalyx (animal) and glycosylated biological membranes. Beyond the variation found in the carbon backbone length of the monomer, anomericity, side group orientation and substitution, the carbohydrate assembly often branches, further complicating structure, because of this diversity, each cell, plasma membrane, or intracellular organelle has a characteristic carbohydrate, which can potentially be recognized by specific lectins. (De Hoff *et al.*, 2009). Carbohydrate residues on the exterior of a cell are often bound by a specific lectin or a small group of lectins. The binding with simple or complex carbohydrate conjugates is reversible and non-covalent. Since phytohemagglutinins are proteins associated in the plant with other protein material, they cannot easily be detected by chemical analysis and are usually determined by their biochemical and biological activities, i.e., hemagglutination and toxicity (Jaffé, 2012)

Hemagglutinating activity is the most commonly used assay for the detection of lectin in a sample due to the simplicity of implementation and ease visualization of agglutination. Aliquot of sample is serially diluted in microtitration plate before addition of erythrocytes and hemagglutinating activity (titer) is defined as the reciprocal of the highest dilution of sample promoting full agglutination of erythrocytes (Jaffé, 2012). The hemagglutinating activity occurs when the lectin binds to carbohydrate from erythrocyte surface promoting a network among them (Plate 2.1A); sometimes lectin is not detected due to steric hindrance in the lectin-carbohydrate interaction (Teixeira-Sa/ *et al.*, 2009). The hemagglutination assay allows the assessment of lectin stability to pH and temperature values and thus can determine the conditions to be used in the biotechnological application of lectin. Additionally the assay may reveal lectin carbohydrate specificity defined by carbohydrate that more effectively inhibits the hemagglutinating activity (Plate 2.1B). The detection of a given hemagglutinin by the agglutination test depends on the use of the right kind of blood cells. Rabbit blood is satisfactory for the nutritionally important agglutinins but is inactive with many other lectins. The hemagglutination test may be very useful if applied correctly because it is simple and no special facilities are required. For ricin, it is not as sensitive and specific as the toxicological assay since there apparently exist interfering factors in mixed feeds (Jaffé, 2012). For the assay of residual toxicity it should be supplemented by animal tests. Feeding experiments have been performed with a wide variety of animals, but rats and chicks are often preferred because of the ease of handling and for economic reasons The specificity of lectins towards carbohydrates can be defined on the basis of 'Hapten inhibition test', in which various sugars or saccharides are tested for their capacity to inhibit the property of hemagglutination of erythrocytes. (Jaffé, 2012).

The binding property of many lectins can be affected by more than one carbohydrate moiety; this is because each lectin molecule possesses two or more carbohydrate-binding sites that are essential for their ability to agglutinate cells or to react with complex carbohydrates (Himansha and Sarathi, 2012). Hapten inhibition test is commonly used for identification of lectin specificity in present day.

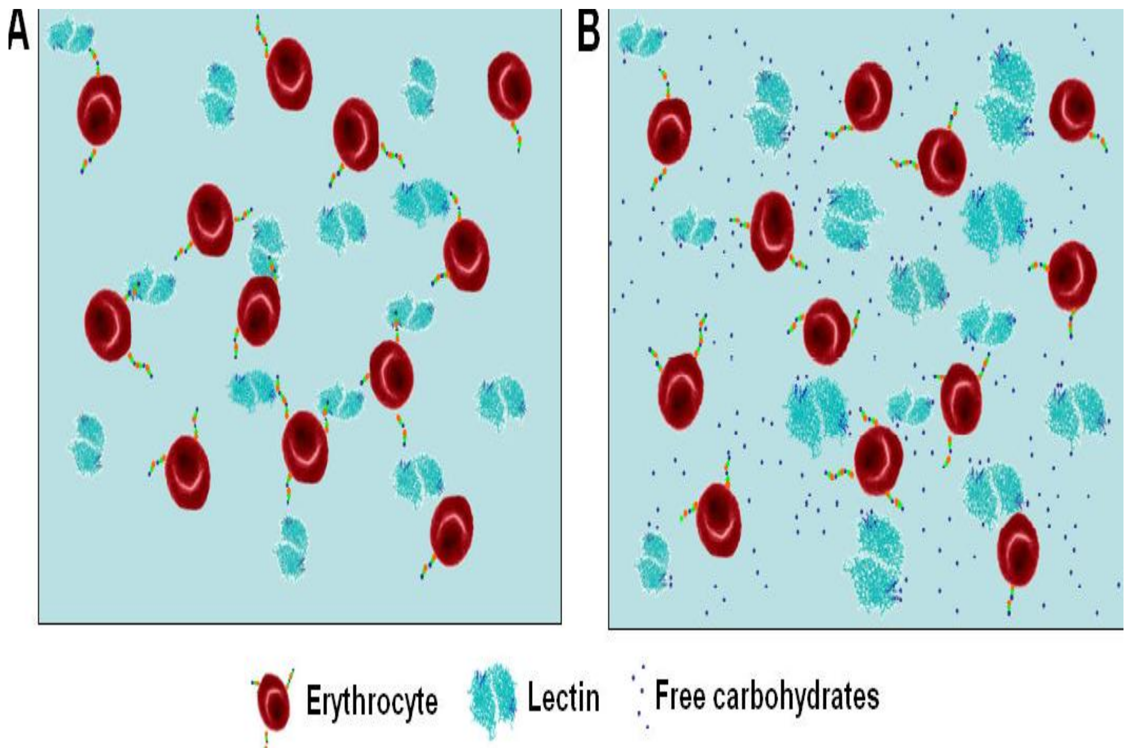


Plate 2.1: Schematic representation of erythrocyte network promoted by lectin binding to surface carbohydrates (A) and inhibition of hemagglutinating activity by free carbohydrates (B).

Titre is the quantity of a substance (extract) required to produce a reaction with a given volume of another substance (erythrocyte) as depicted in Plate 2.2 below. Row 1 shows a positive agglutination reaction up to the sixth well, while row 4 shows a negative agglutination reaction.

Agglutination titre is the highest dilution of the biological sample that still results in agglutination, with no agglutination being observed at any higher dilution.

Titre is an approximation of the lectin activity in each unit volume of a sample.

The term is used in serological reactions and is determined by preparing serial dilutions of lectin to which a constant amount of Red Blood Cell is added.

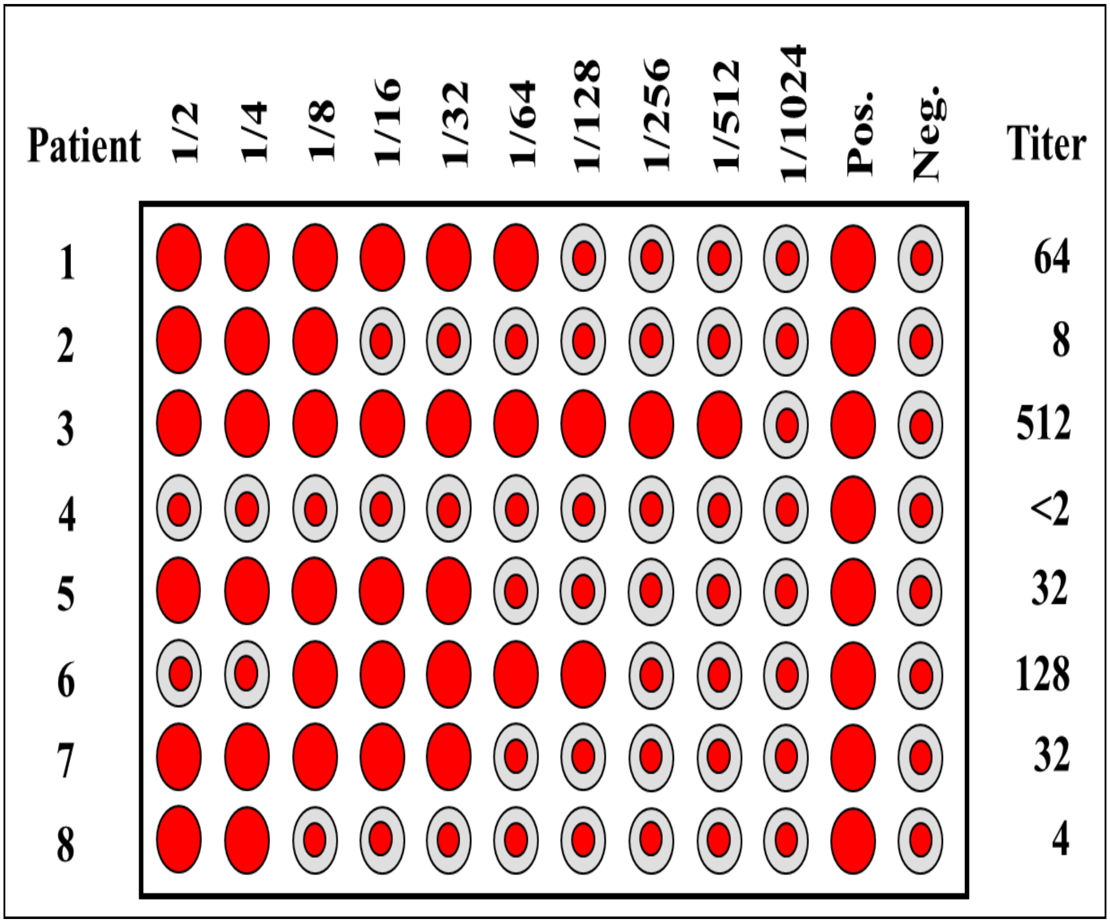


Plate 2.2: **Picture of a Micro Titre Plate after Agglutination Reaction.**

2.6 Polymerase Chain Reaction Assay To Detect Lectin Gene.

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The polymerase chain reaction is a powerful technique that has rapidly become one of the most widely used techniques in molecular biology because it is quick, inexpensive and simple. The technique amplifies specific DNA fragments from minute quantities of source DNA material, even when that source DNA is of relatively poor quality (Mohini and Deshpande, 2010). The basic PCR principle is simple. As the name implies, it is a chain reaction: One DNA molecule is used to produce two copies, then four, then eight and so forth.

This continuous doubling is accomplished by specific proteins known as polymerases, enzymes that are able to string together individual DNA building blocks to form long molecular strands. To do their job polymerases require a supply of DNA building blocks, i.e. the nucleotides consisting of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G). They also need a small fragment of DNA, known as the primer, to which they attach the building blocks as well as a longer DNA molecule to serve as a template for constructing the new strand. If these three ingredients are supplied, the enzymes will construct exact copies of the templates. PCR is a method used to acquire many copies of any particular strand of nucleic acids. It’s a means of selectively amplifying a particular segment of DNA. The segment may represent a small part of a large and complex mixture of DNA (Mohini and Deshpande, 2010).

The isolation of lectins from plants especially legumes is beneficial, and a further line of research involves deducing the molecular weight of the lectins, and after knowing the molecular weight, amino acid sequencing of the lectin can be executed to generate the RNA coding of the protein that could eventually lead to the identification of the specific cDNA sequence. Such cDNA templates could be saved in cDNA libraries. Using these cDNA libraries, primers of the lectins can be formed to understand their properties and biological role at molecular level. Lectins of therapeutics interests can be preserved and can be used in clinical research and healthcare therapies (Mohini and Deshpande, 2010).

2.7 The Plants

2.7.1 *Albizia lebbeck*

Albizia lebbeck, AL is a leguminous plant belonging to the family *Fabaceae* (Formerly *Leguminosae*), member of the subfamily *Mimosae*. AL is large deciduous tree with grayish bark; young shoots glabrous. Leaves are evenly 2- pinnate and the leaflets are in 5-9 pairs, 2.5-5.0 cm long, broadly oblong and pale green, unequal sided, very obtuse glabrous above and reticulately veined beneath. Flowers are stalked, white fragarant in globose umbellate heads 2-3.8cm diameter. Peduncles 3.8-7.5cm long solitary or 2-4 together from the axils of the upper leaves. Calyx 4 mm. long teeth short, Corolla 1 cm long; tube glabrous; lobes 2.5 mm long. Stamens much longer than the corolla. Pod is 10-30 cm long and 2.5-5.0 cm broad, flat straw coloured and contains 4-12 pale brown seeds. Flowering and fruiting periods are April to June. Its vernacular name in hindu is Shirish. Some common names of AL are Hindi- Garso, Siris, Sanskrit- Barhapusph, Bhandi, Kalinga , Urdu- Darash, West Indies- Woman'Tongue, Brazil- Heart-to-black, Ceylon- Kona, English- Parrot tree French- Acacia lebbeck (Mishra *et al.,* 2010). Locally in Nigeria, it is known as "eshegeshege" in Bini language, "igbagbo" in Yoruba and "barankache" in Hausa. (Ehiagbonare and Onyibe, 2008.). Low moisture content makes the shelf life for the seed long. Low lipid content is a favorable factor in preventing rancidity of seeds stored for long periods. The ash contents (7.84%) of this seed is higher than that of other legumes (Mishra *et al.*, 2010).

Phytochemical screening of Albizia lebbeck seeds (El-Mousallamy, 1998) showed that Cyanogenic glycosides 0.11 mg g-1 , Phytic acid 0.25 mg g-1 Oxalate (mg g-1) 2.80 Saponin 18% Tannin 0.002 mg g-1 Saponins are glycosides components often referred to as ‘natural detergent’ because of their foamy nature. It has been established that saponins have anti -carcinogenic activity, immune modulation activities and regulation of cell proliferation as well as health benefits such as cholesterol lowering capacity (Mishra *et al.*, 2010). Tannin reduces protein solubility by forming a complex with protein, thereby causing a reduction in digestibility and hence depressed growth (Seiglar, 1998).

It is grown as fodder for camels, water buffalo and cattle in India and other countries. Goats eat the fallen leaves and flowers. The leaves are reported to be good fodder with 17-26% crude protein; 100 kg of leaves yield 11-12 kg of digestible protein and 37 kg of digestible carbohydrate. It has been reported that rabbits and bats feed on the leaves also (Lowry *et al.*, 2004).

The leaves of AL are remarkably free of toxins and tannins and are low in phenolic compounds. In the area of apiculture its flowers have fragrance and so attract bees. *Albizia lebbeck* is a nitrogen fixing woody legume. The amount of nitrogen fixed, increases with the age of the plant (Kadiata *et al.*, 1996). In addition, the nitrogen rich leaves are valuable as mulch and manure.

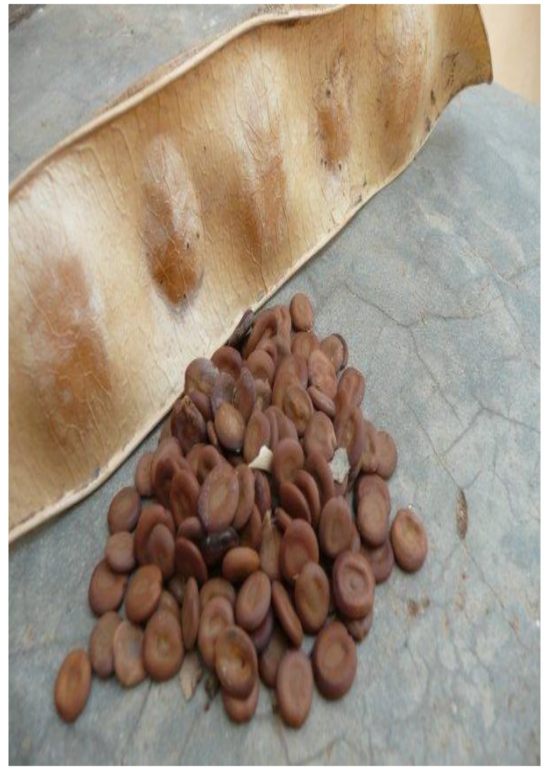
 

Plate 2.3: *Albizia lebbeck* plant, showing leaves, flower, pods and seeds. *Ahmadu Bello University Botanical Garden, 2013.*

It was concluded by Nimish *et al* (2010) that the methanolic extract of AL bark possess strong anti-arthritic and anti-oxidant property while in another study the carcass parameters of boiler chickens fed graded level of Toasted Albizia lebbeck Seed Meal (TASM) were significantly ($p<0.05$)higher than in other treatment in all parameters assessed. This showed that the birds were able to tolerate TASM up to 5% level of inclusion, but beyond this, overall performance carcass characteristics and mortality were affected.

2. 7. 2 *Piliostigma thonningii*

Piliostigma thonningii (PT), is a leguminous plant belonging to the sub family *Caesalpiniacea*, a family that comprises of trees, shrubs or very rarely scramblers. The tree is perennial in nature and its petals are white to pinkish colour produced between November and April. While the fruits, which is a hairy, hard, flattish pod turns rusty brown, woody and twisted which splits at ripening and usually persistent on the tree are produced between June and September (Lock and Simpson, 1999). Locally, the seed is called Abefe in the Yoruba land (Nigeria). Other names include Monkey bread, Camel's foot, Kalgo (Hausa) and Okpoatu (Ibo).

P. thonningii grows in open woodland and savannah regions that are moist and wooded grassland in low to medium altitudes. It is widely distributed in Africa and Asia. It is found growing abundantly as a wild uncultivated tree in many parts of Nigeria such as Zaria, Bauchi, Ilorin, Plateau, Lagos and Abeokuta (Jimoh and Oladiji, 2005).

The level of dietary fibre is quite high when compared with that of most legumes and seeds (Elegbede, 1998).The high crude protein content of *P. thonningii* seed (30.33± 0.31%) coupled with the fact that it is abundant in this part of the world may encourage its use as high protein sources in some food formulations (Jimoh and Oladiji, 2005).The moisture content of P. thonningii (6.71 ± 0.40%) is lower than that of most legume seeds (Giani, 1993; Temple *et al.,* 1991). This implies that the shelf life for this seed will likely be longer than that of most legumes. The ash content of 3.50 ± 0.04% for this seed is comparable with that of other legumes which has been reported to range between 3.0 and 4.8% (Elegbede, 1998). While the carbohydrate content (23.00 ± 0.24%) is at the lower side when compared with that of other legumes ranging from 23% in groundnut to 66% in Bambara groundnut (Ene-Obong and Carnovale, 1992; Apata and Ologhobo, 1994). The phytochemical screening of the seed showed the presence of saponins, flavonoids, phenolics, glycosides, anthraquinones as well as cardiac glycosides; while tannins, steroids, phylobatannins and triterpenes were absent. Some of these chemical compounds have been reported to have inhibitory effects on some gram-negative bacteria such as Escherichia coli and Bacillus subtilis amongst others (Jimoh and Oladiji, 2005). They also have prominent effects on animal systems and microbial cells (Liu *et al.,* 1990; Topcu *et al.,* 1993; Oyagade *et al.,* 1999). The presence of these chemical compounds therefore suggests the pharmacological activities of *P. thonningii*. The seeds of *P. thonningii* fruits have been reported to be eaten by African antelope and elephant while farmers in the lower Savanna region grind up the seed as fodder for cattle during winter months (Jimoh and Oladiji, 2005).

Plate 2.4: *Piliostigma thonningii* showing leaves, pods and seeds. National Research Institute for Chemical Technology, 2013.

CHAPTER THREE

MATERIALS AND METHOD

3.1 Materials

3.1.1 Plant Material

The seeds of *Albizia lebbeck* were collected from the Ahmadu Bello University Botanical Garden and *Piliostigma thonningii* seeds were collected from the compound of National Research Institute of Chemical Technology, Zaria.

The plants were authenticated by a taxonomist at the herbarium section of Biological Sciences Department, Ahmadu Bello University Nigeria. The voucher specimen numbers for *Albizia lebbeck* and *Piliostigma thonningii* seeds, 1486 and 171, respectively were deposited at the herbarium.

3.1.2 Chemicals and Reagents

Sephadex G-75 was purchase from Pharmancia (USA), Sodium dihydrogen phosphate, hydrogen disodium phosphate, sodium chloride, Ammonium Sulphate, Sodium Hydroxide, Ethylenediaminetetraacatic acid (EDTA), dithiothreitol (DTT), 2-mercaptoethanol were purchase from Sigma (USA), Sodium Dodecyl Sulphate, Protein Markers, N,N,N',N'-tetramethylethylenediamine (TEMED) where purchased from Bio-Rad laboratories (USA), Mueller Hinton Agar (MHA) , Potato Dextrose Agar (PDA) purchased from Oxoid (USA), Zymo Research plant/seed DNA kit, Agarose gel, Agarose (0.75 g in 1x TAE BUFFER), Ethidium Bromide (10mg/ml), 1x TAE (0.04M Tris-Acetate, 0.002M EDTA, pH8.0), 6x loading buffer purchased from Thermo Fisher Scientific(USA). All other reagent used were of analytical grade.

3.1.3 Equipment

Ultra Violet Trans-illuminator, microcentrifuge, UV-visible spectrophotometer, electrophoretic machine, Eppendorf centrifuge 5430R, weighing balance, pH meter, incubator, ultracentrifuge, Techgene Amplifier, Power source, UV protected Glasses were used.

3.2 Method

3.2.1 Preparation of sample and Extraction of Lectins from *Albizia lebbeck* and *Piliostigma thonningii* seeds

The dry seeds of A*lbizia lebbeck* and P*iliostigma thonningii* were ground to powder using a blender (Philip blender) and 10grams of the powdered seed was soaked separately in 30mls of 0.06M phosphate buffer saline at 4^{o}C for 4 hours separately. The seeds were ground separately with minimum volume of PBS and the extract were collected in separate 5ml centrifuge tubes and were centrifuged at 7500rpm, at 4^{0}C for 20 minutes. The supernatant were taken after centrifugation and decanted into a measuring cylinder separately. One mililitre of the supernatant was stored in an Eppendorf tube as crude at 4^{0}C for each plant (C_1- Crude *Albizia lebbeck* seed extract and C_2- Crude *Piliostigma thonningii* seed extract) and the remaining were taken for salting out Process (Petnual *et al*., 2010).

3.2.2 Partial purification of lectins

3.2.2.1 *Precipitation/ salting out process of protein using Ammonium Sulphate.*

According to the ammonium sulphate salt chart (appendix I), ammonium sulphate was added to the crude extract (the supernatant) with continuous stirring using a glass rod to 80% saturation. The sample was kept at 4^{O}C for one hour and centrifuged. The precipitate was collected from the suspension by centrifugation at 10000×g for 30minutes. The supernatant was discarded and the precipitate dissolved in minimum volume of PBS and kept at $4^{O}C$. This procedure was carried out for both plants (Petnual *et al*., 2010).

3.2.2.2 *Preparation of the sephadex G75 Matrix column.*

The Sephadex gel G75 was made by weighing out 4.5g of gel and soaked in 100ml of 0.005M phosphate buffer saline of pH 7. It was allowed to swell overnight. After swelling the top layer of buffer was decanted and the gel was loaded into the column gradually to prevent formation of air bubbles. After loading the gel, it was washed with 100ml of buffer and the tap locked, before the introduction of the sample, the absorbance at 280nm (Abs_{280}) was taken using Ultra Violet spectrophotometer and it was <0.005 (Silva *et al*., 2007).

3.2.2.3 *Gel chromatography*

The 80% saturation ammonium sulphate precipitated seed extract was applied to sephadex G75 column which had been washed by PBS solution (pH 7) with an optical density (O.D) value decreasing and tending to zero, the eluted sample was collected in 50 fractions of 2ml each at a flow rate of 400µl per minutes and its O.D was determined at 280nm.The fractions containing lectin, on the basis of Abs_{280} and a detectable hemagglutination activity against chicken erythrocyte were combined. This procedure was carried out for the different plant samples (Silva *et al*., 2007).

3.2.2.4 *Molecular Mass Determination by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis*

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in accordance with the procedure of Laemmli (1970), using a 12% resolving gel and a 5% stacking gel. At the end of electrophoresis, the gel was stained with Coomassie brilliant blue dye. Marker standard proteins and the purified lectin were determined.

3.2.2.5 *Sample Preparation for SDS-PAGE*

The samples (Crude, ammonium precipitate fraction and the purified fractions) were each prepared by adding 20 µl of the loading buffer (2.5ml of 1M Tris pH 6.8, 4ml Glycerol, 0.8g SDS, 2ml β-mercaptoethanol, Bromophenolblue, and 10ml distilled water) to 5 µl of the partially purified lectin protein in a ratio of 1:4. The mixture was boiled for 10min and kept on ice until needed for loading.

3.2.2.6 *Preparation of SDS-PAGE Gels*

The separating gel (12%) was prepared to a final volume of 10 ml by adding; 3.35 ml deionized water, 2.5 ml of 1.5M Tris pH 8.8, 0.1% SDS, 4ml of 30% Bis-acrylamide solution, 50µl of 10% Ammoniumpersulphate (APS) (0.1g/ml; freshly prepared), and 15µl N, N, N', N'-tetramethylethylenediamine (TEMED). The solution was swirled to mix and the mixture was loaded immediately into the gel casting tray of the set up to prevent it from polymerization in the beaker. The stacking gel (4%) was prepared to a final volume of 5ml by adding; 3ml deionized water, 1.25ml of 0.5M Tris pH 6.8, 50µl of 10% SDS, 665µl of 30% acrylamide, 25µl of 10% Ammoniumpersulphate (APS), and 10µl N, N, N', N'-tetramethylethylenediamine (TEMED). It was layered carefully on the separating gel while ensuring there wasn't any bubble formation and allowed to cast (Schagger and von Jagow., 1987).

3.2.2.7 *Loading, Running and Staining of the Sample*

The tank was filled with 1500ml of running buffer working solution consisting of 0.025M Tris (pH 8.3), 1.92M Glycine, and 0.1% SDS. Afterwards, the prepared sample (20µl) was dispensed into the gel wells. Electrophoresis was carried out at 50V for 7hours. The gel was removed and stained overnight with Coomassie blue in a staining tray. The staining solution constitutes the following; 0.1% Coomassie blue G-250 in 50% methanol, and 10% acetic acid. This was followed by rinsing with destaining solution (10% methanol and 10% acetic acid) until the proper destaining of the gel was achieved, the protein bands were then visualized and the molecular weight estimated by comparing the electrophoretic mobility of the sample against the molecular mass marker protein (Schagger and von Jagow., 1987).

3.2.2.8 *Composition of molecular weight marker*

The composition of the molecular weight markers used are; Phosphorylase b 97,400 daltons, Serum albumin 66,200 daltons, Ovalbumin 45,000 daltons, Carbonic anhydrase 31,000 daltons, Trypsin inhibitor 21,500 daltons and Lysozyme 14,400 daltons.

3.2.3 Determination of protein concentration

Biuret Method

The Biuret method is based on the complexation of Cu^{2+} to functional groups in the protein's peptide bonds (Enechi and Nwabueze, 2013). The formation of a Cu^{2+}-protein complex requires two peptide bonds and produces a violet-colored chelate product which is measured by absorption spectroscopy at 540 nm. Over a given concentration range, the measured absorption at 540 nm is linear with respect to the concentration of total protein.

Standard protein solution of bovine serum albumin (BSA), of 10mg/ml was prepared, by weighing 1g of the BSA and dissolving it in 100ml of distilled water contained in a volumetric flask, sodium chloride was introduced to help the protein dissolve, after which it was poured into a fresh container and kept in the refrigerator at 4˚C.

Biuret reagent was prepared by adding with stirring, 300ml of 10% (w/v) Sodium Hydroxide to 500ml of a solution containing 0.3% copper sulfate pentahydrate and 1.2% sodium potassium tartrate, then diluted to one litre and stored in a dark cupboard.

The standard curve was prepared (Appendix II) and used in determining the concentration of protein in the crude, ammonium sulphate precipitate, and sephadex G75 purified fractions.

3.2.4 Preparation of Chicken Erythrocytes.

Healthy chicken blood (4-5ml) was collected immediately the animal was sacrificed in a 15 ml tube to which the anticoagulant EDTA was previously added and washed with normal saline to obtain the erythrocytes. The washed erythrocyte was suspended in 2ml PBS and 5% RBC was prepared (Laija *et a*.l, 2010, Yufang *et al*., 2010).

3.2.5 Haemaglutinnation Assay

The haemagglutination activity of *Albizia lebbeck* lectin and *Piliostigma thonningii* lectin were detected when erythrocytes were added to each. The assay was carried out in a 96 well round bottom microtitre plate. The first well of each row served as positive control to which 50µl of plant sample and 50µl of blood was added and the last well served as negative control which contained 50µl of blood and 50µl of PBS solution. Between the positive and negative control each well contains blood, PBS and lectins. First of all 50ul PBS was added to all the wells. Then 50µl of crude was poured to the first well and it was serially diluted till the negative control. Similar procedure was followed for the other samples and for the other plant. Finally 50µl of 5% washed red blood cells was poured to each well. After that the plate was placed in a plane surface without disturbing it. After two hours the haemagglutination was observed in the wells containing the crude plant extract (Laija *et al*, 2010, Yufang *et al*., 2010).

3.2.6 Antibacterial and Antifungal Assay

3.2.6.1 *Test Organisms*

The test organisms used were clinical isolates of bacteria and fungi obtained from the Department of Microbiology, Ahmadu Bello University, Zaria.

The Isolates include; *Staphylococcus aureus, Bacillus subtilis* (Gram-positive), *Escherichia coli, Salmonella typhi* (Gram-negative), *candida albicans* (Fungus).

3.2.6.2 *Preparation of Different Concentration of the Protein.*

Various concentration of the crude sample ranging from 100% to 12.5% were prepared using sterile distilled water as a diluent (Oliveira *et al*., 2007)

3.2.6.3 *Sensitivity Test of the Protein Using Agar Well Diffusion Method*

The bacterial and fungal isolate were standardized using 0.5 Macfarland turbidity standard. The standardized innocula were uniformly streaked into sterile petri-dishes containing sterile Mueller Hinton Agar (MHA) for bacteria and sterile Potato Dextrose Agar (PDA) for fungi growth.

Four wells were punched on each agar plates with the aid of a sterile cork borer (8mm in diameter). The wells were properly labelled according to the number of different concentrations of the crude prepared (100%, 50%, 25%, and 12.5%). The punched wells were then filled up with the different concentration of the protein. The plates were allowed to stay on the bench for one hour for the protein to diffuse into the agar, after which the plates were incubated at 37^0C for 24hrs for bacteria and the plates of Potato Dextrose Agar for fungi were incubated at room temperature for about 3 days.

After the incubation period, the plates were observed for any evidence of inhibition which will appear as a clear zone that would be completely devoid of growth around the well (Zone of inhibition). The diameter of the zones is usually measured using a transparent ruler calibrated in millimeter (mm) (Henry, 2005).

The same procedure was carried out for the two purified fractions.

3.2.7 DNA Extraction.

DNA Extraction was done using Zymo research (ZR) Plant/Seed kit. Components of the kit and the manufacturer's protocol used are listed in Appendix II. DNA was eluted in 50µl of elution buffer.

3.2.7.1 *Electrophoretic Characterization of DNA by Agarose Gel Electrophoresis.*

3.2.7.1.1 Preparation of Agarose Gel:

According to the Treseder laboratory protocol, 2007, the electrophoretic tank was prepared for gel casting by placing the gel tray in a way that the edges were sealed and the comb is placed near the edges of the tray in a way that the fingers of the comb slightly above the plate, but not touching it. Agarose Gel was made by weighing 0.75g of agarose in a conical flask and added to 50ml of 1x TAE Buffer (Tris-acetate-EDTA). The agarose was solubilized by heating in a microwave oven for 50 seconds, allowed to cool down to 60˚C and 10µl of Ethidium bromide was added, and the whole solution was poured into the taped tray (gel slab) in a horizontal position with the comb inserted at one end before polymerization was completed to make the gel.

When the gel was polymerized, 300ml of a 1x TAE buffer was prepared and poured into the electrophoretic tank, the gel slab was turned horizontally and the combs gently removed. Gel was totally submerged in the buffer but was not covered more than 1cm above the gel.

3.2.7.1.2 loading of samples:

Two microliter of loading dye was mixed with 10µl of Eluted DNA using the pipette and then loaded into the well, power was turned on and ran at 100volts for 45minutes. When the dye front travelled approximately 80% of the gel length, the power source was switched off and the gel tray removed. The ethidium bromide stained bands in the gel were viewed with the UV Trans-illuminator.

3.2.7.1.3 Estimation of the quality and quantity of the extracted genomic DNA by UV Spectrophotometry:

For spectrophotometric analysis, 10µl of DNA sample was diluted with 90µl of TE Buffer (IDTechnologies) and 100µl of the buffer was used as blank. The spectrophotometer reading for the diluted DNA sample was recorded at 260nm and 280nm. The quality and concentration was calculated by imputing these readings in the formula below (Vural, 2010).

$$\text{Adequate purity} = \frac{\text{A260nm}}{\text{A280nm}} = 1.7 - 2.0$$

$$\text{Concentration of DNA } (\mu g/\mu l) = \frac{\text{A260} \times 50\mu g/ml \times \text{dilution factor}}{1000} = X\mu g/\mu l$$

3.2.8 Polymerase Chain Reaction (PCR)

PCR was carried out according the method of Vural, 2010 and Maltas *et al.,* 2011 with some modifications in two tubes of a total volume of 20 µl. The tubes contained 2 µl of genomic DNA, 3.8 µl of primer mix (forward and reverse) (0.5mM of each primer), 10 µl of 10x PCR master mix Buffer, 1.2 µl of 1.5 mmol/l $MgCl_2$, 1.0µl of 5% Dimethylsulfoxide (DMSO) and 2µl of nuclease free water.

The program was initiated on a TechGene amplifier with 5min of denaturation at 94˚C, followed by 40 cycles of amplification with denaturation for 60 sec at 94°C, annealing for 60 seconds at 66°C, and an extension at 72°C for 5 min; followed by a final elongation at 72°C for 5 min.

The primer LEC5/LEC6 which in a previous work amplifies a 180 bp fragment of the lectin gene of soy bean (Vural , 2010) used were;

LEC 5 Forward: **5'-TCAACGAAAACGAGTCTGGTG-3'**

LEC 6 Reverse: **5'-GGTGGAGGCATCATAGGTAAT-3'**

The amplification products (10 µl) were electrophoresed in 1.5% (w/v) TAE agarose gels containing 10mg/ml ethidium bromide. The electrophoresis ran in TAE buffer at 100V for 40minutes and DNA bands were observed under ultraviolet light and photographed by an Image System.

CHAPTER FOUR

RESULTS

4.1 Purification Profile of *Albizia lebbeck* Seed Lectin and *Piliostigma thonningii* Seed Lectin

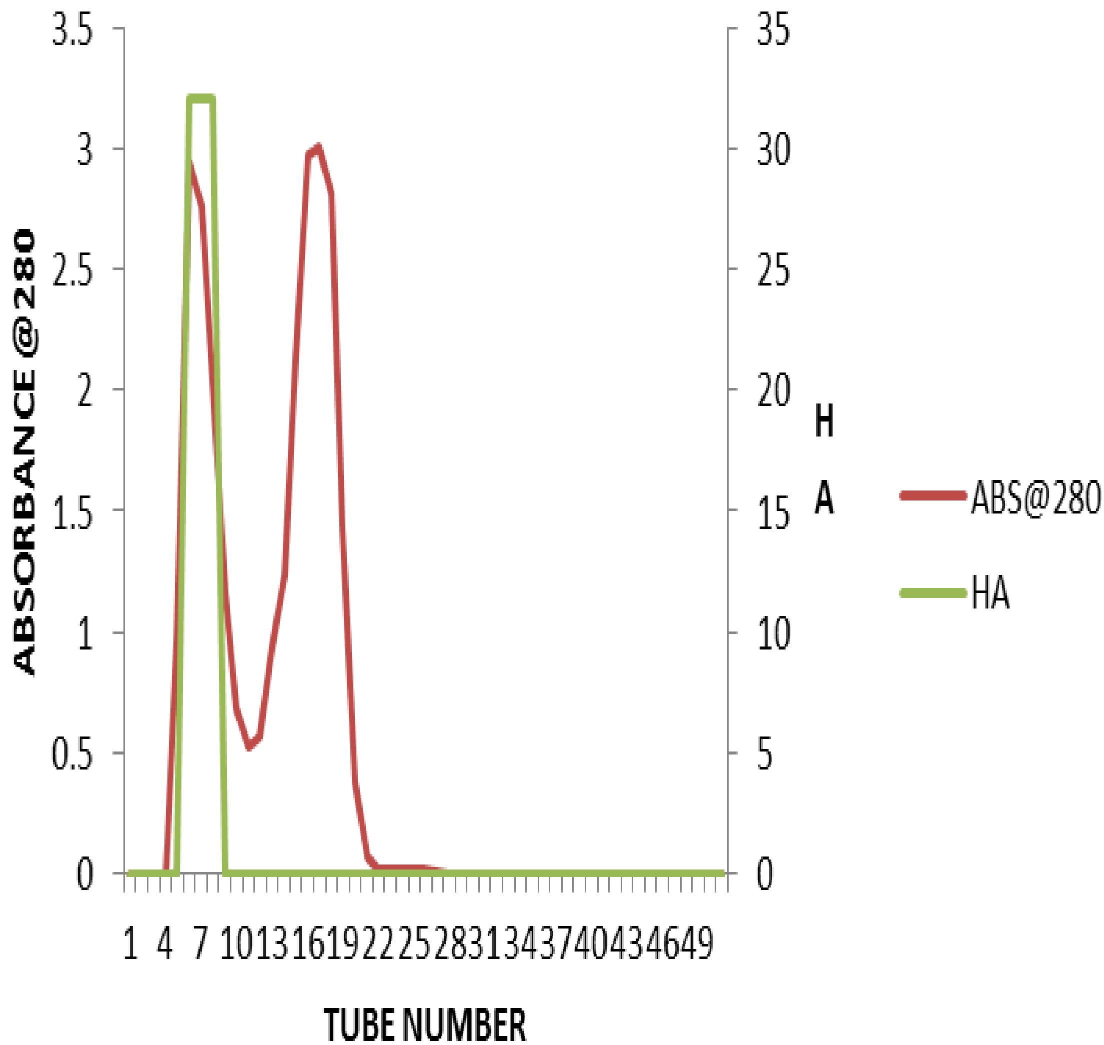
The purification profile for the partial purification of lectin protein from the seeds of *Albizia lebbeck* is summarized in Tables 4.1. The crude protein for *A. Lebbeck* seed contained approximately 9.23 mg/ml total protein with a specific activity of 277.35 HAU/mg. Precipitation of the crude protein with ammonium sulphate resulted to a purification fold of 0.68 and a yield 50 %. The result obtained following gel filtration on Sephadex G-75 column revealed a total yield of 25% lectin with a specific activity of 217.61 HAU/mg.

Figure 4.1 shows the elution profile of the protein lectin from *Albizia lebbeck* after gel filtration on Sephadex G-75 column. From the plot of *Albizia lebbeck*, two peaks was observed, the fractions 5,6 and 7 was pooled together to form *A.lebbeck* purified protein 1(P1) while the fraction 15,16,17 was pooled together to form *A.lebbeck* purified protein 1(P2), Hemagglutination assay was carried out on the fractions collected in both peaks separately and the peak with agglutinating activity (P1) had about 2.941 mg/ml of protein concentration with a total activity of 32 hemagglutinating unit.

Table 4.1: Purification Profile for *Albizia lebbeck* seed lectin protein.

Purification Step	Total Protein (mg)	Total Activity (TITRE)	Specific Activity (TITRE/mg)	Purification Fold	Percentage Yield. %
Crude Protein	9.23	2560	277.35	1.00	100
Ammonium Sulphate Fractionation (80%)	6.77	1280	189.06	0.68	50
Gel Filtration on Sephadex G-75 Column	2.941	640	217.61	0.78	25

One Hemagglutinating unit (HAU) (TITRE) is the highest dilution of 50 ul giving a complete hemagglutinating activity.



KEY:

HA: Hemagglutinating activity per 50µl of lectin.

Figure 4.1: Elution Profile of *Albizia lebbeck* seed lectin on Sephadex G-75 Column Chromatography (Elution rate at 400µl per min).

The purification profile for the partial purification of lectin protein from the seeds of *Piliostigma thonningii* is summarized in Tables 4.2. The crude protein for *P. thonningii* seed contained approximately 3.78 mg/ml total protein with a specific activity of 169.31HAU/mg. Precipitation of the crude protein with ammonium sulphate resulted to a purification fold of 0.72 and a yield 50 %. The result obtained following gel filtration on Sephadex G-75 column revealed a total yield of 50% lectin with a specific activity of 1230.76HAU/mg.

Figure 4.2 shows the elution profile of the protein lectin from *Piliostigma thonningii* after gel filtration on Sephadex G-75 column. From the plot of *Piliostigma thonningii*, two peaks was observed The fractions 6, 7 and 8 was pooled together to form *Piliostigma thonningii* partially purified protein 1(P1) while the fraction 16,17 and 18 was pooled together to form *Piliostigma thonningii* partially purified protein 1(P2), Hemagglutination assay was carried out on the fractions collected in both peaks separately and the peak with agglutinating activity (P2) had about 0.261 mg/ml of protein concentration with a total activity of 16 hemagglutinating unit.

Table 4.2: Purification Profile for *Piliostigma thonningii* seed lectin protein.

Purification Step	Total Protein (mg)	Total Activity (TITRE)	Specific Activity (TITRE/mg)	Purification Fold	Percentage Yield. %
Crude Protein	3.78	640	169.31	1.00	100
Ammonium Sulphate Fractionation (80%)	2.63	320	121.67	0.72	50
Gel Filtration on Sephadex G-75 Column	0.26	320	1230.76	7.27	50

One Haemaglutinating unit (HAU) (TITRE) is the highest dilution of 50 ul giving a complete hemagglutinating activity.

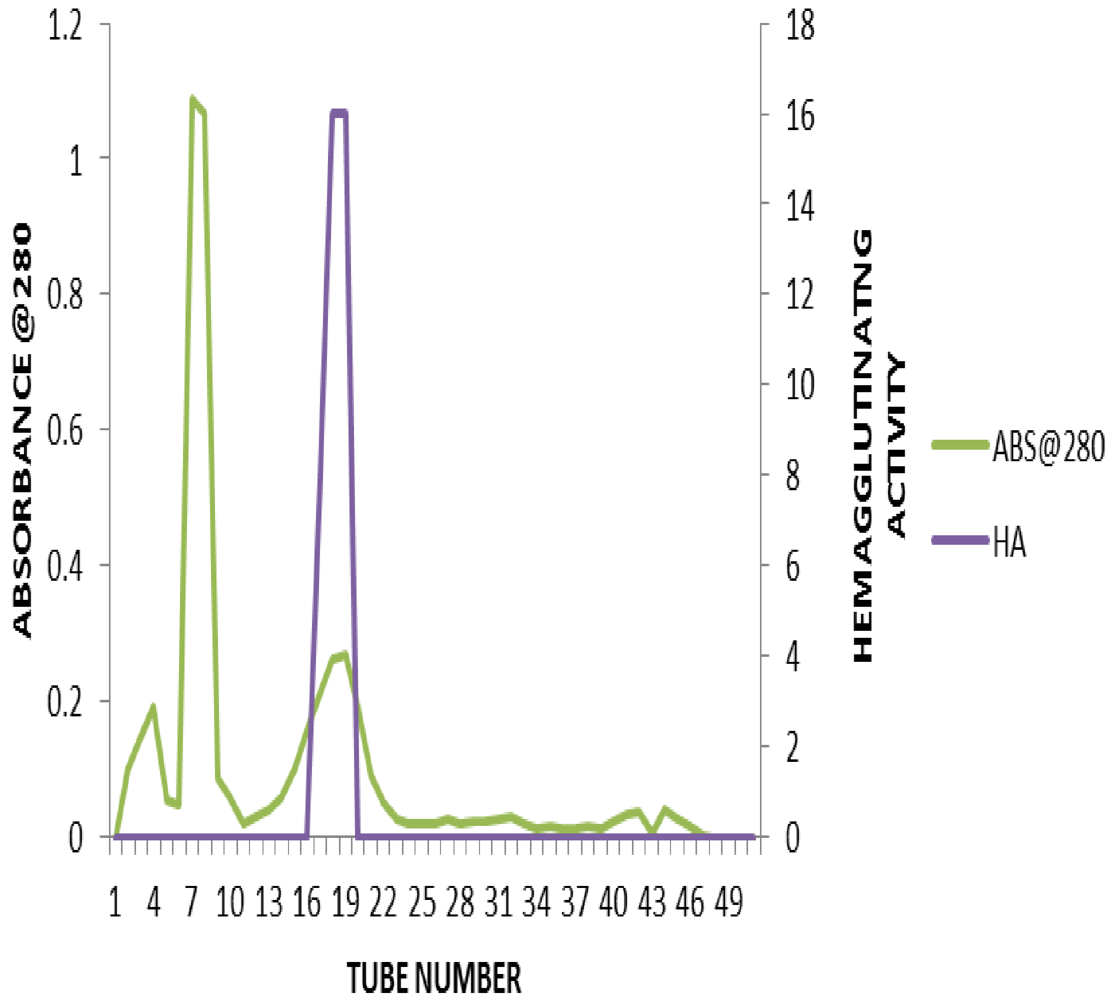


Figure 4.2: Elution Profile of *Piliostigma thonningii* seed lectin on Sephadex G-75 Column Chromatography (Elution rate at 400µl per min)

4.2 SDS- PAGE

4.2.1 Molecular Weight of lectin from Albizia lebbeck seed

The electrophoregram of the sample under denaturing conditions, stained with Coomassie blue is shown in plate 4.1. A band of the protein sample was visualized against the standard protein markers on the gel and the molecular weight was estimated from the plot of log of molecular weight of the standard marker proteins against their respective relative mobility (R_f). The molecular weight of the desired protein was estimated from the standard protein markers (Phosphorylase b 97,400 daltons, Serum albumin 66,200 daltons, Ovalbumin 45,000 daltons, Carbonic anhydrase 31,000 daltons, Trypsin inhibitor 21,500 daltons and Lysozyme 14,400 daltons) to be 21 kDa for *Albizia lebbeck* lectin and no distinctive band was observed for *Piliostigma thonningii* lectin.

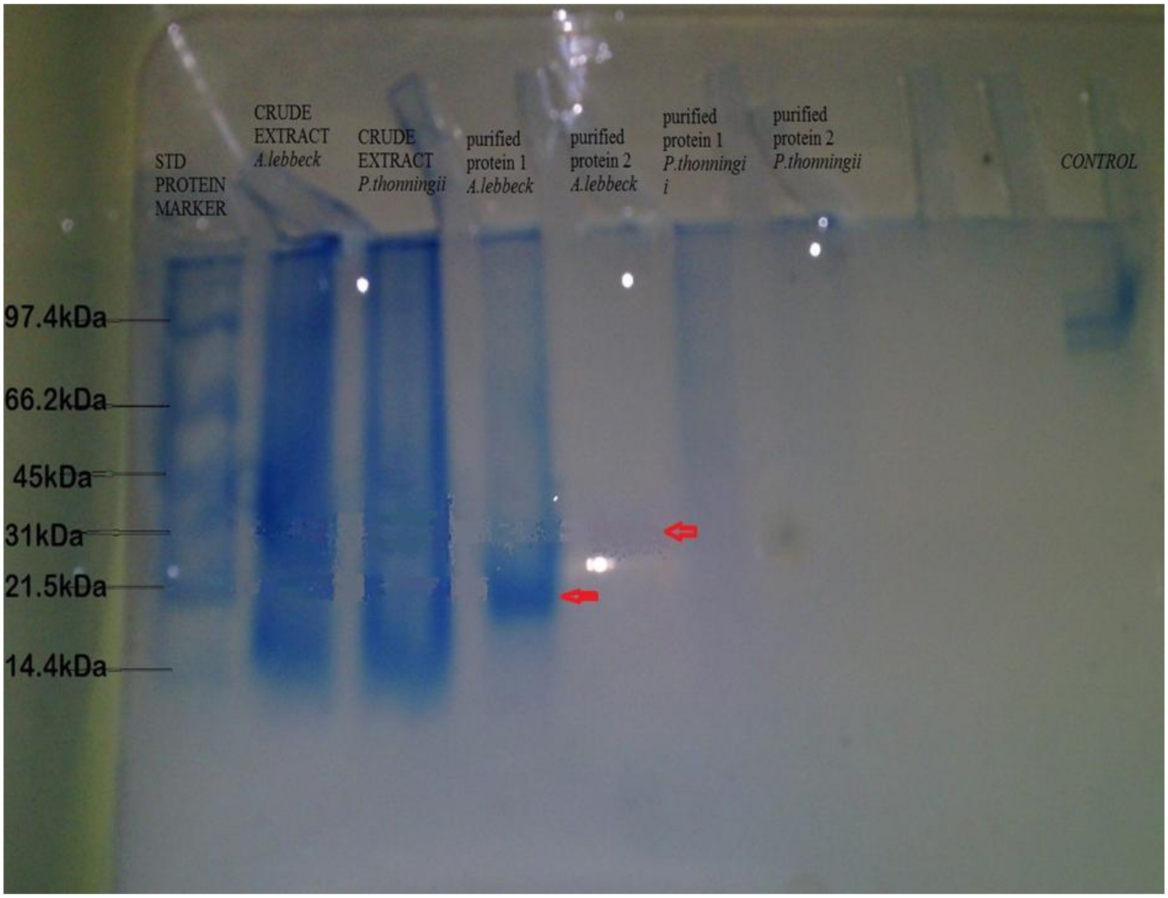


Plate 4.1: Electrophoregram of *Albizia lebbeck* seed lectin on Polyacrylamide gel using Coomassie blue staining. The first Red arrow represents lectin with an estimated molecular weight of **21 kDa**

KEY

Lane1: Standard protein markers

Lane 2: Crude Extract of *Albizia lebbeck.*

Lane 3: Crude Extract of *Piliostigma thonningii.*

Lane 4 and 5: Purified protein *Albizia lebbeck* 1and 2.

Lane 6 and 7: purified protein *Piliostigma thonningii* 1and 2

4.3 Antimicrobial Activity

The result showed the absence of antimicrobial activity against the micro organisms tested. The photomicrograph (4.2a) shows the absence of a zone of inhibition around the well 1,2,3,4 containing the different concentration of the partially purified fractions 1 and 2 (P1and P2) of *Piliostigma thonningii* made with the cork borer in the Petri dish inoculated with *Eschericia coli* labelled EC. The same absence of zone of inhibition was observed for the other organisms *Bacillus subitils* (BC), as shown in figure 4.2b, *Salmonella typhi* (ST) shown in figure 4.2d, and *Staphylococcus aureus* (SA) shown in figure 4.2d. Figure 4.2e shows the the photomicrograph with no zone of inhibition around the well for the fungi *Candida albicans* labelled CAND. The minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) were not determined since there was no zone of inhibition observed during sensitivity test.

Plate 4.2(a): *Escherichia coli* (EC) purified fraction P1 and P2 for the plant *Piliostigma thonningii.*

Plate 4.2 (b): *Bacillus subitilis* (BC) purified fraction P1 and P2 for the plant *Piliostigma thonningii.*

Plate 4.2 (c): *Salmonella typhi* (ST) purified fraction P1 and P2 for the plant *Piliostigma thonningii*.

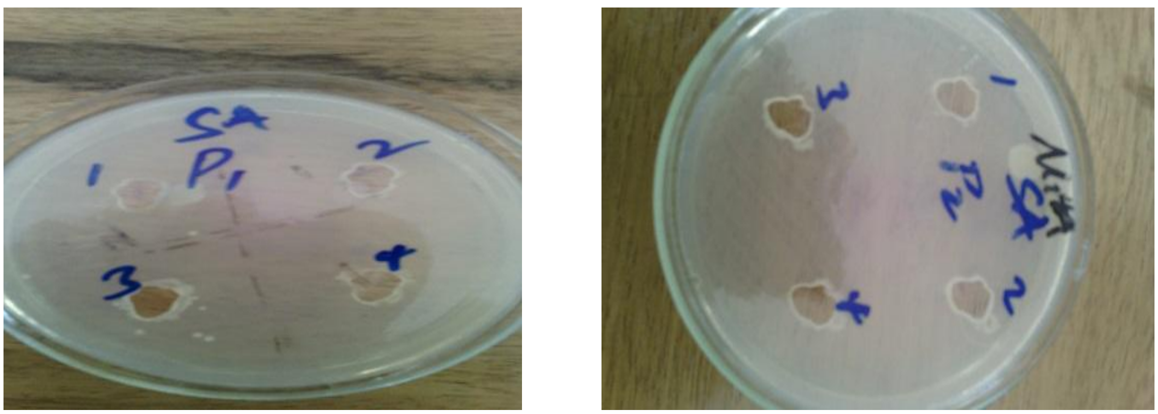


Plate 4.2 (d): *Staphylococcus aureus* (SA) purified fraction P1 and P2 for the plant *Piliostigma thonningii*.

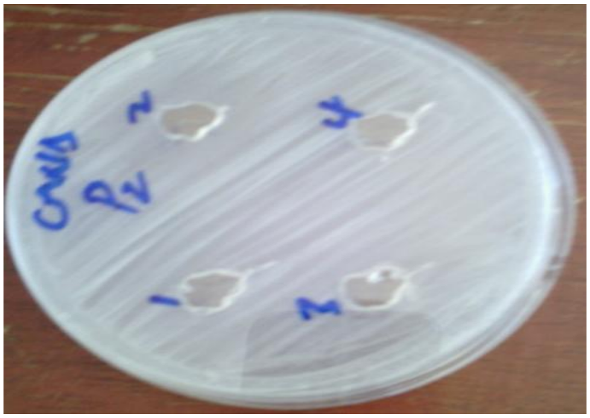
 

Plate 4.2 (e): *Candida albicans* (CAND) purified fraction P1 and P2 for the plant *Piliostigma thonningii.*

Key:

BC-*Bacillus subitilis* SA- *Staphylococcus aureus*

EC- *Escherichia coli* ST- *Salmonella typhi*

CAND- *Candida albicans*

P_1- Purified protein fraction 1

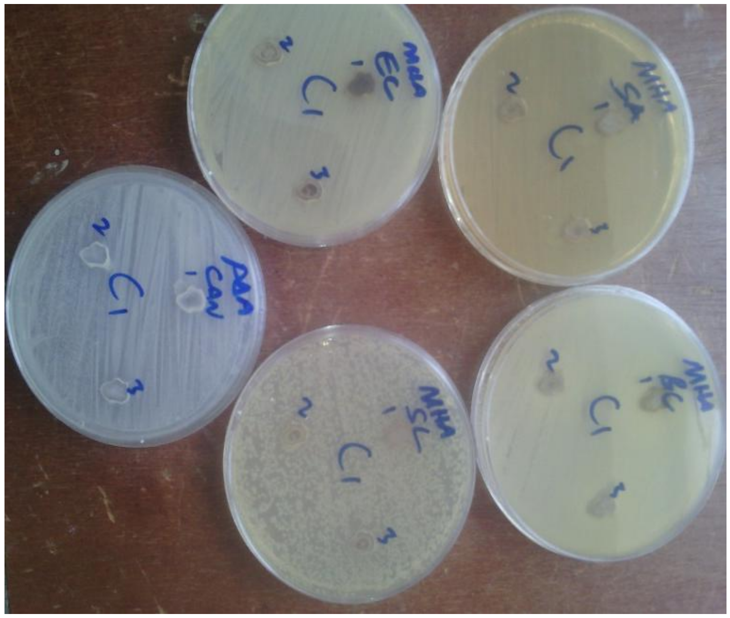
P_2 –Purified protein fraction 2

MHA- Mueller Hinton Agar

PDA- Potato Dextrose Agar

The result presented in plate 4.3 is the photomicrograph that shows that there was no zone of inhibition observed around the well labelled 1,2,3 for the crude extract of *Albizia lebbeck,* C1 shown in plate 4.3A against the organisms tested labelled as follows *Escherichia coli* (EC), *Bacillus subitilis* (BC), *Staphylococcus aureus* (SA), *Salmonella typi* (SL) on Muella Hinton agar (MHA) and *Candida albicans*(CAN) on Potato Dextrose agar (PDA).

The result presented in plate 4.3B is the photomicrograph that shows that there was no zone of inhibition observed around the well labelled 1,2,3 for the crude extract of *Piliostigma thonningii,* C2 against the organisms tested labelled as follows *Escherichia coli* (EC), *Bacillus subitilis* (BC), *Staphylococcus aureus* (SA), *Salmonella typi* (SL) on Muella Hinton agar (MHA) and *Candida albicans*(CAN) on Potato Dextrose agar (PDA).



A

B

Plate 4.3: Photomicrograph showing the absence of antimicrobial activity for the various test bacteria for the crude samples of *Albizia lebbeck* (C1) as seen in plate A and *Piliostigma thonningii* C2 as seen in plate B.

The result presented in plate 4.4 is the photomicrograph that shows that there was no zone of inhibition observed around the well labelled 1,2,3 for the partially purified fractions P1 and P2 gotten from gel filtration as observed from the elution profile of *Albizia lebbeck.* Plate 4.4A is the result observed for purified fraction P1 against the organisms tested; labelled as follows *Escherichia coli* (EC), *Bacillus subitilis* (BC), *Staphylococcus aureus* (SA), *Salmonella typi* (SL) on Muella Hinton agar (MHA) and *Candida albicans*(CAN) on Potato Dextrose agar (PDA).

The result presented in plate 4.4B is the photomicrograph that shows that there was no zone of inhibition observed around the well labelled 1,2,3 for the partially purified fractions (P2) of *Albizia lebbeck.* against the organisms tested labelled as follows; *Escherichia coli* (EC), *Bacillus subitilis* (BC), *Staphylococcus aureus* (SA), *Salmonella typi* (SL) on Muella Hinton agar (MHA) and *Candida albicans*(CAN) on Potato Dextrose agar (PDA).

A B

Plate 4.4: Photomicrograph showing the absence of antimicrobial activity around the point of sample introduction for the *Albizia lebbeck* purified fractions P1 (plate A) and P2 (plate B).

Key:

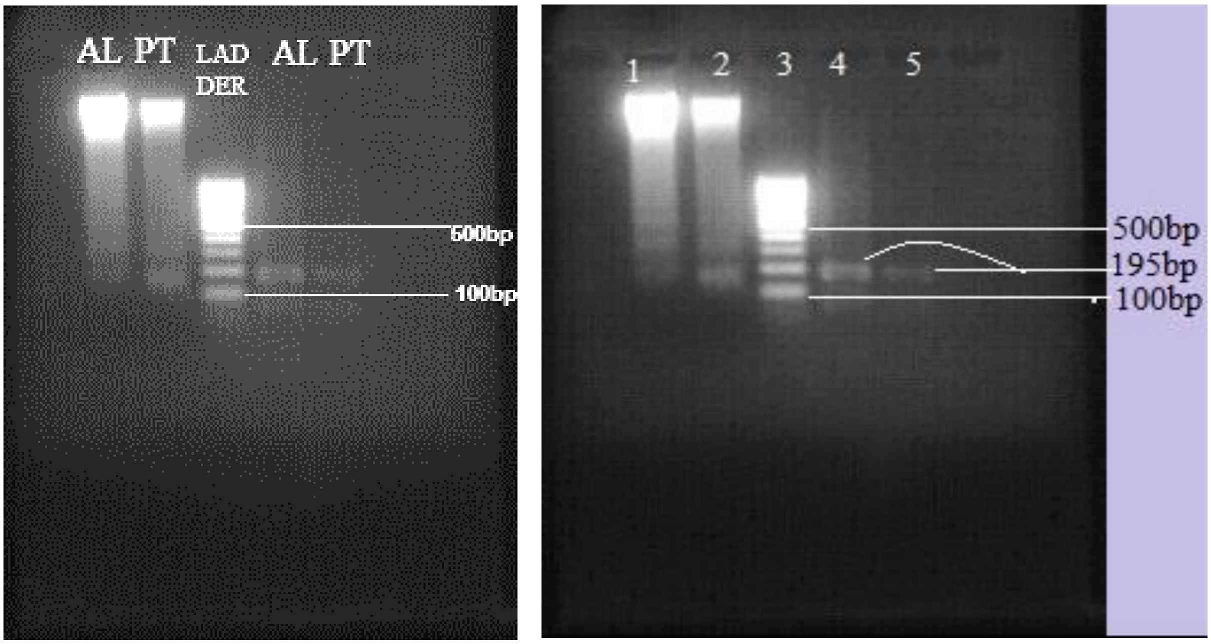
BC-*Bacillus subitilis* SA- *Staphylococcus aureus*

EC- *Escherichia coli* SL- *Salmonella typhi*

CAN- *Candida albicans*

4.4 DNA Isolation and Polymerase Chain Reaction.

The result shown in Plate 4.5 is the genomic DNA (gDNA) of *Albizia lebbeck* and *Piliostigma thonningii* represented in the first and second well respectively, both plant gDNA were of adequate quality, quantity (Appendix vi)and purity for applying polymerase chain reaction (PCR). The amplification of gene of interest (lectin) using the LEC5/LEC6 Primer pair yielded a 195 bp product which is represented in lane4 for *Albizia lebbeck* and lane 5 for *Piliostigma thonningii*. Lane 3 contains the 1000kb DNA ladder from which the size of the amplicons gotten for both plant gDNA was estimated.



A B

Plate 4.5: Both plates (A and B) represents the agarose gel electrophoresis of LE5/LE6 gene PCR products on a 1.5% Agarose gel. Lane 1: genomic DNA from *A.lebbeck*, lane 2: genomic DNA from *P.thonningii*, lane 3: DNA ladder, lane 4: *A.lebbeck* amplicon of lectin gene, lane 5: *P.thonningii* amplicon of lectin gene.

KEY:

AL *Albizia lebbeck*

PT *Piliostigma thonningii*

CHAPTER FIVE

DISCUSSION

5.1 Partial Purification and Molecular Weight Determination.

In this study, precipitation of the lectin with ammonium sulphate resulted in a 0.68-fold purification for *A. lebbeck* and 0.72-fold for *P. thonningii,* this low purification fold may be as a result of the ammonium sulphate salt affecting the hemagglutinating ability of the lectin protein or an instability as a result of separating the protein from solution. Further purification was achieved by size exclusion chromatography on sephadex G-75. Upon SDS PAGE, the partially purified *Albizia lebbeck* lectin (ALL), fraction with hemagglutinating activity (P1) yielded a polypeptide band of 21kDa,while for that of partially purified *Piliostigma thonningii* lectin (PTL), the protein fraction P1 and P2 , gave no distinctive polypeptide band, and this could be because the fraction pooled was too diluted, efforts to concentrate it while trying to preserve its tertiary structure still did not result in any band, a more sensitive staining method like the Silver staining could be used alongside concentrating the protein.

Maricel *et al.,* (2004) reported the purification of lectin from *Moringa oleifera* seeds on a 2-step purification process, gel filtration using Sephadex G-75 column gave an approximate molecular Weight of 12,200 Da for the isolated lectin which is lower than the molecular weight reported in this study, though the purification process is the same, the molecular weight difference may be as a result of the specie differences. Lectin purified from seeds of *Caesalpinoideae* plant, *Bauhinia variegata,* as reported by Pinto *et al.,* (2008) by affinity chromatography on lactose-agarose, analysis by MALDI-TOF spectrometry indicated a MW of 32 817 Da. The results weren't similar to those found for the lectin from *Caesalpinoideae*, *P. thonningii*, a member of this family in this study. In another study (Andreia *et al.*, 2011) , *Swartzia laevicarpa,* a member of the sub-family *Faboideae,* resulted in a lectin fraction that was examined by electrophoresis, and a major protein band with an apparent molecular mass between 27 and 30 KDa was observed.

5.2 Antimicrobial Properties

The antimicrobial activity test of lectin protein was measured against selected bacteria *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Bacillus subitilis* and a fungus, *Candida albicans*. The antimicrobial activities of the lectin protein at different dilution for both plants are shown in Plates 4.2, 4.3, and 4.4. No inhibition zones was observed for both purified plant lectin at the different lectin concentration. The crude plant sample was also measured against the same micro-organisms as shown in Plate 4.3, and gave no inhibition zones showing that both plants seed lectins have no antimicrobial property against the selected micro-organism. In a previous research (Rahul *et al*., 2010) it was reported that the successive ethyl acetate extract of *Albizia lebbeck* leaves are found to have inhibitory effect against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus cereus*, the extract showed sensitivity for both gram positive and gram negative bacteria with maximum against *Pseudomonas aeruginosa* and minimum against *Escherichia coli.* this is different from the result gotten from this study because the lectin studied is from the leaves and the anti microbial activity observed could be as a result of a phytochemical as the lectin protein was not purified but an ethyl acetate extract was used. It has been proposed (Qadir *et al.*, 2013) that the proteins with antibacterial action form a channel on cell membrane and the cell dies as a result of the out flowing of cellular contents, this mechanism being different from that of antibiotics. This channel that is formed on the membrane is as a result of the specificity of lectins for carbohydrates, it could be that the absence of a zone of inhibition in this study is as a result of the lectin protein not being specific for any of the carbohydrate moiety on the cell membrane of the tested micro organisms and so didn't lead to the disruption of the cell membrane and eventual death of the organism.

Antibacterial activity on Gram-positive and Gram-negative bacteria occurs through the interaction of lectin with components of the bacterial cell wall including teicoic and teicuronic acids, peptidoglycans and lipopolysaccharides; study revealed that the isolectin I from *Lathyrus ochrus* seeds bind to muramic acid and muramyl dipeptide through hydrogen bonds between ring hydroxyl oxygen atoms of sugar and carbohydrate binding site of lectin and hydrophobic interactions with the side chains of residues Tyr^{100} and Trp^{128} of isolectin I (Bourne *et al.,* 1994).

There are only a few lectins known to possess antifungal activity such as the lectins from the seeds of *Phaseolus vulgaris* and *Pisum sativum*, from the pepper seed *Capsicum frutescens* and from the mushroom *Astragalus mongholicus* (Ye *et al.,* 2001; Yan *et al.*, 2005; Ngai and Ng, 2007and Sitohy *et al.*, 2007).The inhibition of fungal growth can occur through lectin binding to hyphas resulting in poor absorption of nutrients as well as by interference on spore germination process (Lis and Sharon, 1981), the lectin of *Pisum sativum* seeds inhibited the growth of *Aspergillus flavus*, *Fusarium oxysporum* and *Trichoderma viride* (Sitohy *et al.,* 2007), the authors suggested the use of lectin for clinical microbiology and therapeutic purposes.

This study provides conclusive evidence that *Albizia lebbeck* seed lectin and *Piliostigma thonningii* seed lectin has no antimicrobial activity against the micro organisms tested.

5.3 Amplification of Lectin Gene

DNA quality is determined by its fragment length and its degree of damage due to exposure to heat, low pH and/or nucleases that cause hydrolysis, depurination and/or enzymatic\degradation. Therefore, DNA quality varies according to the material under examination, the degree of processing of the sample and the applied DNA extraction method. The plants seed used were dried and proper precautions were taken towards its preservation before its use. The DNA of both plant were successfully isolated using the Zymo Research Plant/Seed Kit as it had the crushing beads that assisted in the crushing of the plant cell wall exposing the plant nucleic material and also the reagents were prepared by the manufacturer which helped in reducing to a large extent any interference from other inhibitory materials, agarose gel electrophoresis of the DNA isolated using ethidium bromide staining indicated a DNA of good integrity, good enough for polymerase chain reaction. A sensitive qualitative molecular detection was developed using Polymerase Chain Reaction, an optimized amplification reaction that was carried out on both plant seed gDNA using the primer LEC5(Forward) **5'-TCAACGAAAACGAGTCTGGTG-3'**and LEC6 (Reverse) **5'-GGTGGAGGCATCATAGGTAAT-3'** produced a 195 bp product confirming the presence of lectin gene in both plant seed. The primer pairs LEC5/LEC6 was reported for identification of the galactose specific lectin gene present in soybean (Vural, 2010; Maltas *et al.,* 2011), because both plants used in this study were legumes of the same family with soybean the same primer with a little optimization of reaction condition produced an amplification product of 195 bp which indicates an evolutionary relationship, and a more sensitive and specific method of identification. Although the 195bp amplicon size is supposed to translate for a protein of an approximate molecular weight of between 119- 126kDa, the molecular weight after SDS-PAGE gotten in this study for the fraction with hemagglutinating activity after gel filtration on *Albizia lebbeck* seeds extract resulted in a protein with an estimated molecular weight of 21kDa, this difference observed could be related to the different phases in the life cycle of the plant, the process of translating the genetic information in the mRNA to protein and the possible loss of amino acid due to the post translational modification carried out on the protein (wang *et al*., 2004). The N-terminal amino acid sequence and subsequent full length cDNA sequence may be able to shed more light on this observed difference.

The present study on the molecular detection and development of the protocol for the optimization of the primer pair conditions is the first report on *Piliostigma thonningii* and *Albizia lebbeck* growing in Nigeria.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

- Lectin from *Piliostigma thonningii* and *Albizia lebbeck* were purified to 7.27 and 0.78-fold respectively and a yield of 50% and 25% on Sephadex G-75 column with a final specific activity of 1230.76 HAU/mg and 217.61 HAU/mg respectively.

- Haemaglutination activity was confirmed using Chicken Erythrocyte, and same reaction maintained throughout the study to monitor the lectin haemaglutinating ability throughout the partial purification procedure.

- SDS-PAGE of the lectin protein from *Albizia lebbeck* revealed a molecular weight of 21 kDa. *Piliostigma thonningii* showed no distinctive band on the electrophoregram.

- The antimicrobial activity of the lectin proteins measured against selected bacteria; *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Bacillus subitilis* and a fungus, *Candida albicans* showed an absence of inhibitory activity; indicating no antimicrobial activity by the lectins in the seed of both plants against the selected microorganisms.

- Amplification reaction using the primers Lec5 (forward) / Lec6 (Reverse) under optimized reaction conditions produced a product of 195 bp in both plants, the first step towards development of their genetic map.

6.2 **Conclusion**

This study has provided evidence to the presence of lectin in *Piliostigma thonningii* and *Albizia lebbeck* from Nigeria using the hemagglutination reaction on chicken erythrocyte and an optimized polymerase chain reaction was developed for the detection of the lectin gene in both plants using the Lec5/Lec6 primer pair that previously amplified a galactose specific lectin gene in soybean. A partial purification and the determination of the molecular weight of *Albizia lebbeck* seed lectin was estimated to be 21 kDa. This study has also provided evidence to the absence of anti-bacterial and anti-fungal properties of the lectins in the seeds extract of *Albizia lebbeck* and *Piliostigma thonningii*.

The amplification of lectin gene in both plants confirmed the report of Vural, 2010; that lectin gene can be identified using specific and sensitive technique like polymerase chain reaction .

Finally, these findings provide information that will initiate more molecular studies on *Albizia lebbeck* and *Piliostigma thonningii* leading to other possible applications.

6.3 Recommendation

Further molecular studies should be carried out to provide better characterization and profiling of the lectin.

REFERENCES

Allen, A.K., Bolnell, G.P., Brown, D.S., Sidebottom, C. and Slabass, A.R. (1996).Potato lectin: a three-domain glycoprotein with novel hydroxyproline-containing sequences and sequence similarities to wheat-germ agglutinin. International Journal of Biochemistry and Cell Biology 28, 1285-1291.

Andreia, V. F., Márcio V., Ramos, J. F., Gonçalves, C., Paulo, A. C. Maranhão, L. R. Chevreuil and Luiz Augusto G. S. (2011). Seeds of Amazonian Fabaceae as a source of new lectins. *Brazillian Journal of Plant Physiology*, 23(3), 237-244.

Apata, D.F and Ologhobo, A.D. (1994). Biochemical evaluation of some Nigerian legumes seeds. *Food Chemistry*, 49, 333-338.

Banerjee, S., Vishwanath, P., Cui, J., Kelleher, D.J., Gilmore, R, Robbins, P.W and Samuelson, J. (2007) The evolution of *N*-glycan-dependent endoplasmic reticulum quality control factors for glycoprotein folding and degradation. Proc Natl Acad Sci USA 104, 11676– 11681.

Barira, I and Asad U. K. (2012). Lectins: To Combat Infections, Protein Purification, Dr. Rizwan Ahmad (Ed.), ISBN: 978-953-307-831-1, InTech, Available from: http://www.intechopen.com/books/proteinpurification/lectins-to-combat-with-infections.

Beintema, J. J (1994). Evolution of arthropod hemocyanins and insect storage proteins,

Molecular Biology Evolution, 11, 493-503.

Benedito, V.A., Torres-Jerez, I., Murray, J.D., Andriankaja, A., Allen, S., Kakar, K., Wandrey, M., Verdier, J., Zuber, H., Ott, T., Moreau, S., Niebel, A., Frickey, T., Weiller G., He, J., Dai, X., Zhao, P.X., Tang, Y., and Udvardi, M.K (2008). A gene expression atlas of the model legume *Medicago truncatula*. *Plant Journal*, 55, 504–513

Bohlool, B.B. and Schmidt, E.L. (1974) Lectins: a possible basis for specificity in the Rhizobium-legume root module symbiosis. *Science*, 188, 269–271.

Bourne, Y., Ayouba, A., Rouge, P and Cambillau, C. (1994). Interaction of a legume lectin with two components of the bacterial cell wall. *The Journal of Biological Chemistry*, 269, 9429-9435.

Boyd, W.C and Shapleigh. E. (1954). Specific precipitating activity of plant Agglutinins(Lectins) . *Science*, 119(3091), 419.

Brill, L. M., Evans, C. J. and Hirsch, A. M. (2001) Expression of MsLEC 1- and MsLEC 2-antisense genes in alfalfa plant linescauses severe embryonic developmental and reproductive abnormalities. *Plant Journal,* 25: 453-461.

Brewin, N.J, and Kardailsky, I.V. (1997). Legume lectins and nodulation by Rhizobium. *Trends*, 2:92-98.

Chrispeels, M.J. (1984) Biosynthesis, processing, and transport of storage proteins and lectins in cotyledons of developing legume seeds. *Philos Trans R Soc Lond Biol,* 304:309–322.

Chrispeels, M. J. and Raikhel, N.V. (1991): Lectins, lectin genes, and their role in plant defense. *Plant Cell,* 3: 1–9.

Collinge, D.B., Kragh, K.M., Mikkelsen, J.D., Nielsen, K.K., Rasmussen, U., Vad, K (1993) Plant chitinases. *Plant Journal*,3:31-40.

Debray, H., Coddeville, B., Bomfin, L.R., and Ramous,M.V. (2009). Asimple micro-method for determining precise oligosaccharidic specificity of mannose-binding lectin. *Glycobiology* 19(12): 1417-1426.

De Hoff, P. L., Laurence M. B, Hirsch, A.M (2009). Plant lectins: the ties that bind in root symbiosis and plant defense, *Molecular Genetics and Genomics*, 1-15, DOI: 10.1007/s00438-009-0460-8.

De-Mejia, E.G., and Prisecaru, V.I (2005). Lectins as bioactive plant proteins : a potential in cancer treatment. *Critical Review of food Science and Nutrition* 45: 425-445.

Desoignies N. (2008). Étude des interactions virus-vecteur:transmission par *Myzus persicae* d'un virus sur le mode non-persistant (PVY) et d'un virus sur le mode persistant (PLRV). Mémoire DEA : Université Catholique de Louvain (Belgique).

Díaz, C.L., Logman, T.J.J., Stam, H.C., and Kijne, J.W (1995). Sugar-binding activity of pea lectin expressed in white clover root hairs. *Plant Physiology,* 109:1167–1177

Drickamer, K. (1988) Two distinct classes of carbohydrate recognition domains in animal lectins. *Journal for Biological Chemistry*, 263:9557–9560.

Edelman, G. M., Cunningham, B. A., Reeke, G. N., Becker, J. W., Waxdal, M**.** J. and Wang, J. L. (1972) The covalent and three-dimensional structure of concanavalin A, *Proceedings of the Nutritional Academy of Science. USA 69,* 2580- 2584.

Ehiagbonare, J.E. and Onyibe, H.I. (2008). Studies on raising and preparation of planting stock of three indigenous forest timber species in Nigeria. *International journal of Biological and chemical science*, 2(4): 573-578.

Elegbede J.A (1998). *Legumes. In: Nutritional quality of plant foods*. Osagie AU, Eka OU (Eds). Post Harvest Research Unit, University of Benin. pp. 53-83.

Enechi, O.C and Nwabueze, E.(2013). A new colorimetric method for the determination of protein. *Advances in Biological Research*, 7(5): 159-162.

Ene-Obong, H.N, Carnovale, E. (1992). A comparison of the proximate, mineral and amino acid composition of some known and lesser known legumes in Nigeria. *Food Chemistry,* 43: 169-175.

El-Mousallamy, A.M.D (1998). Leaf flavonoids of *Albizia lebbeck*. *Phytochemistry*, 48(4): 759-761.

Fitches, E., Woodhouse, S.D., Edwards, J.P., Gatehouse, J.A. (2001). In vitro and in vivo binding of snowdrop (Galanthus nivalis agglutinin; GNA) and jackbean (Canavalia ensiformis; Con A) lectins within tomato moth (Lacanobia oleracea) larvae; mechanisms of insecticidal action. *Journal of Insect Physiology*, 47:777-787.

Florin, L., Carmen, L., Pop Arieta., Marilena, L., Eugen, A., Cosmin, B., Horia, M., (2011). Effect of plant lectin and extract on adhesion molecules of endothelia progenitors. *Central European Journal of Biology*, 6: 330-341.

Garner, O.B, Baum, L.G. (2008). Galectin–glycan lattices regulate cellsurface glycoprotein organization and signalling. *Biochem Soc Trans* 36:1472–1477

Giani, S.Y. (1993). Effect of processing on the proximate composition and functional properties of cowpea (*Vigna unguiculata*) flour. *Food. Chemistry*, 47: 153-158.

Gray, S. M. and Gildow F. E. (2003). Luteovirus-aphid interactions. *Annual. Review. Phytopathology*, 41: 539-566.

Hardman, K.D. and Ainsworth, C.F. (1972). Structure of *concanavalin A* at 2.4-A resolution. *Biochemistry,* 11: 4910–4919.

Habibi, J., Backus, E.A., Czapla, T.H. (1993). Plant lectins affect surviva1 of the potato leaf hopper (Homoptera:Cicadellidae). *Journal of Econmic Entomology,* 86: 945-951.

Habibi, J. E., Backus, E. A. and Huesing, J. E. (2000). Effects of phytohemagglutinin (PHA) on the structure of midgut epithelial cells and localization of its binding sites in western tarnished plant bug Lygus hesperus Knight. *Journal Insect Physiology*, 46(5): 611-619.

Henry, D.I. (2005). *Clinical microbiology procedures hand book*. American Society for Microbiology, Washington DC.

Hester, G., Kaku H., Goldstein I.J. and Wright C.S. (1995). Structure of mannose-specific snowdrop lectin is representative of a new plant lectin family, *Nat. Struct. Biology*, 2 ,472–479.

Himansha .S. And Sarathi.S.P. (2012). Insight of Lectins- A review. *International Journal of Scientific & Engineering Research*, 3(4): 1-9.

Hogenhout, S. A., Ammar, E. D., Whitfield, A. E., Redinbaugh M.G. (2008). Insect vector interactions with persistently transmitted viruses. *Annual. Review Phytopathology*., 46: 327- 359.

Irimura, T and Osawa, T. (1972). Studies on a hemagglutinin from *Bauhinia purpura* alba seeds; *Arch. Biochem. Biophys*. 151 **:**475–482.

Jaffé. W. G. (2012). Hemagglutinin. In: Liener. I (Ed). *Toxic Constituent of Plant Food Stuffs.* Academic Press Incorporated, New York, pp 69-101.

Jimoh, F.O. and Oladiji, A.T. (2005). Preliminary Studies on *Piliostigma thonningii* seeds: Proximate analysis, mineral composition and phytochemical screening. *African Journal of Biotechnology,* Vol. 4 (12) : 1439-1442.

Kadiata, B. D., Mulongoy, K., Isirimah, N. O. (1996). Time course of biological nitrogen fixation, nitrogen absorption and biomass accumulation in three woody legumes. *Biological Agriculture & Horticulture,* 13 (3): 253-266.

Kapoor, C., Vaidya, S., Kaur, H., Jain, A (2014). Role of lectins in Clinical settings. *Clinical Cancer Investigation Journal,* 3: 472-477.

Kieliszewski, M.J., Showalter A.M., Leykam, J.F.(1994), Potato lectin: a modular protein sharing sequence similarities with the extension family, the hevein lectin family, and snake venom disintegrins, *Plant Journal,* 5: 849–861.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature.* 227 : 680-685.

Laija, S.N., Mahesh, S., Smitha, L.S., Remani,P. (2010). Isolation and partial characterization of two plant lectins. *Current Research Journal of Biological Science*. 2(4): 232-237.

Lam, S. K., Ng, T.B (2011). Lectins: production and practical applications. *Applied Microbiology and Biotechnology,* 89: 45-55.

Lis, H and Sharon, N. (1981) Lectins in higher plants. In: Marcus A, (Ed). *The Biochemistry of Plants* vol. 6. New York, NY: Academic Press, pp371-447.

Liu, S., Babajide, O., Charles, D.H., Alvie, M. (1990). 3-methoxysampangine, a Noval antifungal copyrine alkaloids fungi deistopholis pattern. *Agents and Chemotherapy*, 34(4): 529-533.

Lock, J.T, Simpson, M.J. (1999). *Legumes of West Tropical Asia*. 3rd ed. Academic Press Inc, London. pp. 216-220.

Lord, J.M. (1985) The structure and synthe of plant lectins. Tansley. Rev No 3. *New Phytology*, 101:351–366.

Loris, R., Casset, F., Bouckaert, J., Pietinckx,J., Dao-Thi, M., Poortmans, F., Imberly, A., Perez, S.,Wyns, L. (1994). The Monosaccharide binding site of Lentil Lectin: An X-ray and molecular modelling study. *Glycoconjugate Journal*. 11(6): 507-517.

Lowry, J. B., Prinsen, J. H., Burrows, D. (2004). *Forage tree legumes in tropical agriculture: Albizia lebbeck - a promising forage tree for semiarid regions*. Pp. 75-83.

Maltas, E., Nagari, N., Vural, H.C., Salili, Y. (2011). Biochemical and molecular analysis of soybean seed from Turkey. Journal of Medicine and Plant Research. 5(9): 1575-1581.

Mandal, D.K., Kishore, N. and Brewer, C. F. (1994) Thermodynamics of lectin–carbohydrate interactions—titration microcalorimetry measurements of the binding of N-linked carbohydrates and ovalbumin to concanavalin A. *Biochemistry*, 33:1149–1156.

Maricel, D., De Mfsa., Elniikr-Rico, E., Mojica., Florinia E M. (2004). Purification of lectin from mature seeds of malunggay (moringa pterygosperma). *Philippine Journal of Crop Science*, 29(3) :13-24.

McMahon, S.A., Miller, J.L., Lawton, J.A., Kerkow, D.E., Hodes, A., Marti- Renom, M.A., Doulatov S., Narayanan E., Sali A., Miller, J.F., Ghosh P. (2005). The C-type lectin fold as an evolutionary solution for massive sequence variation. *Natural Structural Molecular Biology*, 12:886–892.

Mishkind, M.L, Raikhel, N.V, Palevitz, B.A, Keegstra, K. (1982). Immunocytochemical localization of wheat germ agglutinin in wheat. *Journal Cell Biology*, 92:753–764.

Mishkind, M.L, Palevitz, B.A., and Raikhe1, N.V. (1983). Localization of wheat germ agglutinin-like lectins in various species of the Gramineae. *Science*, 220, 1290-1292.

Mishra, S.S., Gothecha, V.K., Sharma, A. (2010). *Albizia lebbeck*: A short Review. *Journal of Herbal medicine and toxicology*, 4(2):pp. 9-15

Mohini J and Deshpande J.D (2010). Polymerase Chain Reaction: Methods, Principles and Application. International Journal of Biomedical Research, 1 (5) pp. 81- 97.

Ngai, P.H., Ng, T.B. (2007). A lectin with antifungal and mitogenic activities from red cluster pepper (*Capsicum frutescens*) seeds. *Applied Microbiology Biotechnology*, 74:366–371.

Nimish, L. P., Natvarlal, J., Patel, S. B., Kasture, N. P., Jivani,Y. S., Bhalodia, S. V. M. (2010). Free radical scavenging activity of Albizia lebbeck methanolic extract in arthritic rats. *International Journal of Pharmaceutical Research and Development*, 1: 1-6.

North, S.J., Von Guntens., Antonopoulos, A., Trollope, A., Macglashan,D.W. Jr., Jang-lee, J., Delll,A., Metcalfe,D.D., Kirshenbaum,A.S., Bochner, B.S., Haslam, S.M. (2012). Glycomic analysis of human mast cells eosinophils and basophils. *Glycobiology*. 22 : 12-22.

Oliveira, M.D.L., Andrade, C.A.S, Santos-Magalhaes, N.S., Coelho, L.C.B.B., Teixiera, J.A, Carneiro-da-Cunha, M.G., Correia, M.T.S. (2007). Purification of a lectin from *Eugenia uniflora* L. seeds and its potential antibacterial activity. *Letters in Applied Microbiology*, 46: 371-376.

Oyagade, J.O., Awotoye, O.O., Adewumi, T.J., Thorpe, H.T. (1999). Antibacterial activity of some Nigerain medicinal plants. I. Screening for antibactieral activity. *Bioscience. Research. Comm*, 11(3): 193-197.

Petnual. P., Sangvanich. P., and Karnchanatat. A. (2010). A lectin from Rhizomes of Tumeric (*Curcuma longa L*.) and its Antifungal, Antibacterial and alpha-Glucosidase Inhibitory activities. *Food Science Biotechnology* 19(4), 907-916.

Peumans, W.J. and Van Damme, E.J.M. (1996). Prevalence, biological activity and genetic manipulation of lectins in foods. *Trends Food Science Technology*. 7: 132–138.

Peumas, W.J., Winter, H.C., Bemer, V., Van Leuven, F., Goldstein, I.J., Andtrufabach, P. (1997). Isolation of a novel plant lectin with an unusual specificity from *Calystegia sepium. Glycoconjugate Journal* 14 : 259-265.

Peumans, W.J and Van Damme, E.J. (1998). Plant lectin: Versatile protein with important perspective in Biotechnology. *Biotechnology and Genetic Engineering Review*, 15(1) : 199-228.

Pinto, L.S, Nagano, C.S., Oliveira, T.M., Moura, T.R., Sampaio, A.H., Debray, H., Pinto V.P., Dellagostin, O.A., Cavada B.S. (2008) Purification and molecular cloning of a new galactose-specifi c lectin from *Bauhinia variegata* seeds; *Journal of Biological science*, 33: 355–363.

Powell, K. S., Gatehouse, A. M. R., Hilder, V. A., Gatehouse J. A., (1993). Antimetabolic effects of plants lectins and fungal enzymes on the nymphal stages of two important rice pests, *Nilaparvata lugens* and *Nephotettix cinciteps*. *Entomology. Exp. Appl*, 66(2):119-126.

Pusztai A**.** (1991) *Plant lectins*, Cambridge University Press, Cambridge.

Pusztai, A., Ewen, S.W.B., Grant, G., Peumans, W.J., Van Damme, E.J.M., Coates, M.E. and Bardocz, S. (1992). Lectins and also bacteria modify the glycosylation of gut surface receptors in the rat. *Glycoconjugate Journal* 12, 22±35.

Qadir, S., Hussain, I., Rafiq, S., Ganie, A.S., Masood, A., Hamid, R. (2013). Evaluation of antimicrobial activity of a lectin isolated and purified from *Indigofera heterantha. Advances in Bioscience and Biotechnology*, 4, 999-1006.

Raikhel, N. V., Lee, H.I. and Broekaert, W. F. (1993). Structure and function of chitin-binding proteins, Annual. Review. *Plant Physiology and Molecular Biology,* 44, 591-615.

Rahul, C., Pankaj, P., Sarwan, S.K., Mahesh, J.K. (2010).Phytochemical screening and antimicrobial activity of *Albizzia lebbeck. Journal for Chemical and Pharmaceutical Research 2(5), 476-484*

Sato, S., Nakamura, Y. and Kaneko, T. (2008) Genome structure of the legume, *Lotus japonicus*. *DNA Research*, doi:10.1083/dnares/dsn008

Savithiry, S. N., Pastor-Corrales, M.A., Farooq, H.K. and Wesley, M.G (2013). Proteomic analysis of common bean (*Phaseolus Vulgaris L.*) by two-dimensional gel electrophoresis and mass spectrometry. *Journal of Basic and Applied Science* 9: 424-437.

Schagger, H., and von Jagow, G. (1987). Tricine-sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis for the seperation of proteins in the range from 1 to 100kDa. *Analytical Biochemistry,* 166, 368-379.

Schmid , M., Davison, T.S., Henz, S.R., Pape, U.J., Demar, M., Vingron, M., Schölkopf, B., Weigel D., Lohmann, J.U (2005). A gene expression map of *Arabidopsis thaliana* development. *Natural Genetics* 37:501–506

Seigler, D.S. (1998). Plants with saponins and cardiac glycosides. *www.lifwe.vinc.edu/plantbio/363/saponinslides*

Silva, J.A., Damico, D.C.S., Baldasso, P.A., Mattioli, M.A., Winck,F.V., Fraceto, L.F., Novello, J.C., and Marangoni, S.(2007). Isolation and biochemical characterization of a galactoside binding lectin from *Bauhinia variegata candida* seed. *Protein journal*. (26), 191-210.

Sitohy, M., Doheim, M. and Badr, H. (2007).Isolation and characterization of a lectin with antifungal activity from Egyptian *Pisum sativum* seeds. *Food Chemistry*, 104, pp971-979.

Sharon, N. and Lis, H. (1993). Carbohydrates in cell recognition, *Science,* 268:82-89.

Sharon, N. (2007). Lectin: Carbohydrate-specific reageny and biological Recognition molecules. *The Journal of Biological Chemistry*, 282: 2753-2764

Shyamlal, S. Y., Galib, P. K., Prajapati, C. R., Harisha. (2011). Pharmacognostical Screening and Phytochemical Evaluation of Albizia lebbeck Benth. Heartwood. *Asian Journal of Biomedical and Pharmaceutical Sciences* 1 (5), 01-06.

Sumner, J.B. and Howell, S.F.(1936). The identification of the hemagglutinin of the jack bean with *concanavalin A. Journal Bacteriology*; 32:227-237.

Teixeira, E.H., Arruda, F.V., Santiago do Nacimento., Carneiro, V.A., Nagano, G.S., Rocha da Silva, B., Sampaio, A.H., Cavada, B (2012). Biological application of plant and Algae lectins: An Overview. http// dx.doi.org/10.5772/50632.

Teixeira-Sa, D.M.A., Reicher, F., Braga, R.C., Beltramini, L.M., Moreira, R.A. (2009). Isolation of a lectin and galactoxyloglucan from *Mucuma sloanei*. *Phytochemistry*,70:1965-1972.

Temple, V.J., Odewumi, L., Joseph, K. (1991). Soybeans and soybean based diets. In: Proceedings of the 3rd regional workshop on rural development, Jos, 31st July – 2nd Aug 1991. pp. 45-50.

Topcu, G., Sevil, O, Hui-ling, S, Geoffery A. (1993). Cordell Job, Pezzuto M, Candan Bozok-Johansson. Cytotoxic and antibacterial seiguiterphenes from *Viula graveolens*. *Phytochemistry* 33(25): 407- 410.

Treseder laboratory Protocols. (2007). *Gel Electrophoresis: Molecular Techniques.* School of Biological Sciences,University of California, Irvine. Pp 1-8.

Van Damme, E.J.M., Peumans W.J., Barre, A. and Rouge, P. (1998). Plant lectins: a composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. Critical Review. *Plant Science*. 17: 575–692.

Van Damme, E.J.M., Nausicaa, L., Peumans, W.J. (2008). Plant Lectins. In: Kader, J.C., Delseny M (Ed) *Advances in botanical research,* vol 48. Elsevier Ltd, San Diego, pp 107–209

Van Rhijn, P., Goldberg, R.B. and Hirsch, A.M. (1998). *Lotus corniculatus* nodulataion specificity is changed by the presence of a soybean lectin gene. *Plant Cell*, 10:1233-1250.

Vitale, A. and Bollini, R. (1995). Legume storage proteins. In: Kigel, J. and Galili, G. (Ed), *Seed Development and Germination*. Dekker M. Inc, New York, Basel, Hong Kong, pp. 73–102.

Vodkin, L.O., Rhodes, P.R. and Goldberg, R.B. (1983). A lectin gene insertion has the structural features of a transposable element. *Cell*, 34 1023-1031.

Vural, H.C. (2010). Genetic identification of soybean [*glycine max* (l.) Merr.]Growing in turkey for molecular breeding using molecular markers. *Biotechnology. & Biotechnology*, 24(3), 2004-2008.

Wang, S., Zhong, F., Zhang, Y., Wu, Z., Lin, Q., and Xie, L. (2004). Molecular characterization of a new lectin from the Marine Alga *Ulva pertusa. Acta Biochemical et Biophysical sinica*, 36(2), 111-117.

Wang, Y., Wu, S.L., and Hancock, W.S. (2006) Monitoring of glycoprotein products in cell culture lysates using lectin affinity chromatography and capillary HPLC coupled to electrospray linear ion trap-Fourier transform mass spectrometry (LTQ/FTMS). *Biotechnology Progress*, 22, p873 880.

Wen, F., VanEtten, H.D., Tsaprailis, G., Hawes, M.C. (2007). Extracellular proteins in *Pisum sativum* L. root tip and border cell exudates. *Plant Physiology*, 143:773–783.

Yan, Q., Jiang, Z., Yang, S., Deng, W., Han, L. (2005). A novel homodimeric lectin from *Astragalus mongholicus* with antifungal activity. *Archives of Biochemistry and Biophysics*, 442:72-81.

Ye, X.Y., Ng, T.B., Tsang, P.W.K., Wang, J. (2001). Isolation of a homodimeric lectin with antifungal and antiviral activities from red kidney bean (*Phaseolus vulgaris*). *Journal of Protein Chemistry,* 20**:** 367–375**.**

Yufang, H., Yubao, H., Liu, Y., Guang, Q. and Jichang, L. (2010). Extraction and purification of a lectin from Red Kidney bean and preliminary immune function studies of the lectin and four chinese herbal polysaccharides. *Journal of Biomedicine and biotechnology*. 2010, 1-9.

APPENDICES

APPENDIX 1

Ammonium Sulphate Purification Table

This table indicates the grams of solid ammonium sulphate (at 25°C) to be added to one litre of solution to produce a desired change in the presence saturation of ammonium sulphate. Saturated solution is 4.1 M and required 767 grams of salt per litre. This table was based on Green and Hughes in Methods Enzymology. 1, 67-90 (1955). How to use ammonium sulphate concentration table: Select your initial concentration of ammonium sulphate in percent from saturation from the left column, then select required final concentration of ammonium sulphate in percent from saturation from the top row. Intersection will give you and exact amount of ammonium sulphate (NH_2SO_4) in grams for 1 litre of initial solution.

%	10	15	20	25	30	33	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	56	84	114	144	176	196	209	243	277	313	351	390	430	472	516	561	610	662	713	767
10		28	57	86	118	137	190	183	216	251	288	326	365	406	449	494	540	592	640	694
15			28	57	88	107	120	153	185	220	256	294	333	373	415	459	506	556	605	657
20				29	59	78	91	123	155	189	225	262	300	340	382	424	471	520	569	619
25					30	49	61	93	125	158	193	230	267	307	348	390	436	485	533	583
30						19	30	62	94	127	162	198	235	273	314	356	401	449	496	546
33							12	43	74	107	142	177	214	252	292	333	378	426	472	522
35								31	63	94	129	164	200	238	278	319	364	411	457	506
40									31	63	97	132	168	205	245	285	328	375	420	469
45										32	65	99	134	171	210	250	293	339	383	431
50											33	66	101	137	176	214	256	302	345	392
55												33	67	103	141	179	220	264	307	353
60													34	69	105	143	183	227	269	314
65														34	70	107	147	190	232	275
70															35	72	110	153	194	237
75																36	74	115	155	198
80																	38	77	117	157
85																		39	77	118
90																			38	77
95																				39

APPENDIX II

Hemagglutination Assay

To characterize the desired protein, Hemagglutination Assay was performed by using 5% chicken erythrocyte suspension. The assay was carried out in 96 well U- bottom micro titer plate by serially diluting the sample and allowing it to incubate for 2 hours. The following result was observed for both plants. The results showed that the G75 sephadex purified fraction for *Alibizia lebbeck* and *Piliostigma thonningii* retained agglutination till the 5th well (32 titre) and 4th well (16 titre) respectively indicating the presence of lectin but in low concentration.

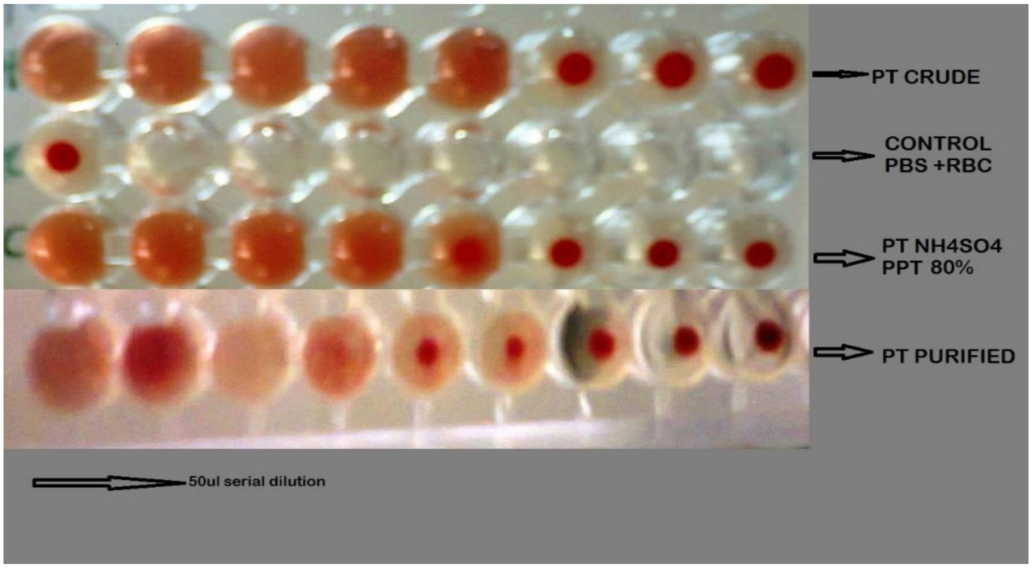
KEY: + positive, agglutination - Negative, no agglutination

HAU = highest dilution giving complete haemaglutination

Result for *Piliostigma thonningii,* using 5% RBC

Sample WELLS (50ul dilution of sample across)

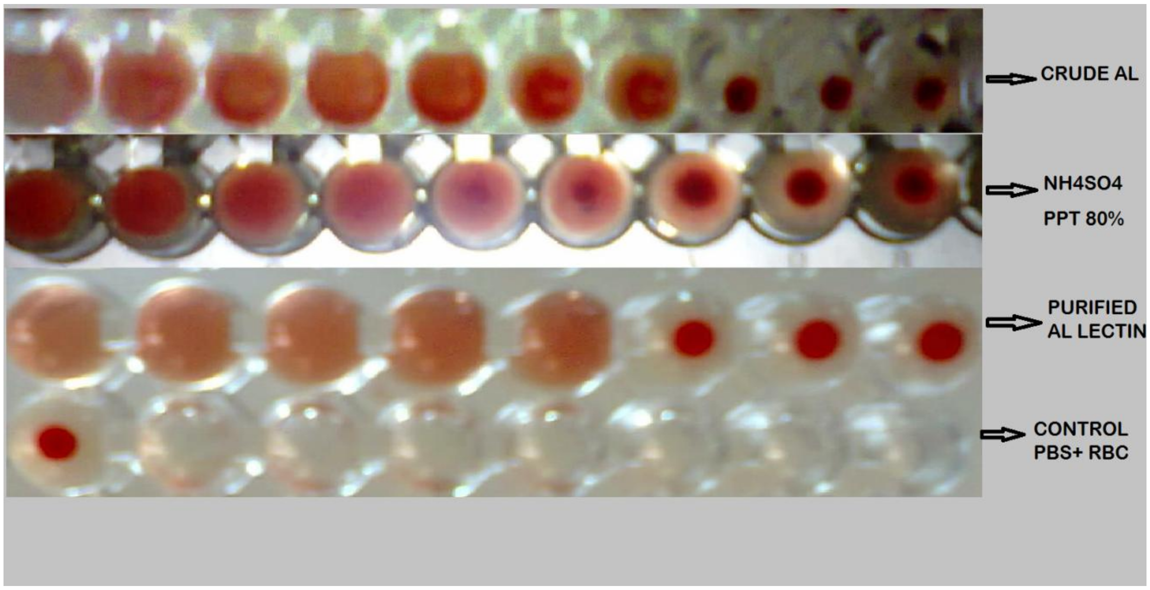
	2	4	8	16	32	64	128	256	HAU
Crude(PT)	+	+	+	+	+	-	-	-	1:32
Control	-	-	-	-	-	-	-	-	NEG
NH_4SO_4 PPT (80%)	+	+	+	+	-	-	-	-	1:16
G75 Sephadex gel filtration	+	+	+	+	-	-	-	-	1:16



Agglutination activity of *Piliostigma thonningii* lectin protein observed in microtitre plate (as viewed from the bottom of the plate).

Result for *Alibizia lebbeck,* using 5% RBC

Sample	WELLS (50ul dilution of sample across)								
	2	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**HAU**
Crude(AL)	+	+	+	+	+	+	+	-	1:128
Control	-	-	-	-	-	-	-	-	NEG
NH_4SO_4 PPT (80%)	+	+	+	+	+	+	-	-	1:64
G75 Sephadex gel filtration	+	+	+	+	+	-	-	-	1:32



Agglutination activity of *Albizia lebbeck* lectin protein observed in microtitre plate (as viewed from the bottom of the plate).

APPENDIX III

Zymo Research Plant/Seed kit Components and Protocol

Components of the kit includes:

- Collection tubes
- Zymo-spin IV-HRC Spin Filters(green tops)
- ZR BashingBead lysis Tube
- Zymo-spin IV Spin Filter(Orange top)
- Zymo-Spin IIC Column
- Lysis solution
- Plant/seed DNA Binding Buffer
- DNA Pre-Wash Buffer
- Plant/seed DNA Wash Buffer
- DNA Elution buffer

Protocol

Before starting

- Beta mercaptoethanol was added to the plant/seed DNA Binding buffer to a final dilution volume of 0.5%(v/v) i.e, 500µl per 100ml.
- The zymo-spin IV-HRC Spin Filters(green tops) was prepared prior to its use by snapping its base, inserting it into a collection tube and spinning in a microcentrifuge at 8000×g for 3minutes.

1) Added 150g of seed sample (already ground using mortar and pestle) into the ZR BashingBead lysis Tube and then added 750µl of the lysis solution to the tube.
2) The cells of the sample was disrupted by the bashing beads by placing the bashing bead lysis tube on the vortex machine for 20minutes.
3) After disruption the ZR Bashing Bead Lysis tube was placed in a micro centrifuge and spinned at 10000×g for 1minute.
4) Transferred 400µl of the supernatant to a zymo-spin IV Spin Filter(Orange top) in a collection tube and centrifuged at 10000×g for 1minute.
5) The filter column was discarded and 1200µl of the plant/seed DNA Binding Buffer was added to the filtrate in the collection tube from step 4 and mixed by pipetting up and down for 5 times.
6) Transferred 800µl of the mixture from step 5 to a Zymo-Spin IIC Column in a Collection tube and centrifuged at 10000×g for 1minute.

7) Discarded the flow through and repeated the step with what was remaining.

8) Added 200µl DNA Pre-Wash Buffer to the Zymo-Spin IIC Column in a new collection tube and centrifuged at 10000×g for 1minute.

9) Added 500µl plant/seed DNA Wash Buffer to the Zymo-Spin IIC Column and centrifuged at 10000×g for 1minute.

10) Transferred the zymo-spin IIC Column to a new collection tube and added 100µl of DNA Elution buffer directly to the column matrix. Centrifuged at 10000×g for 30seconds to elute the DNA.

11) Transferred the eluted DNA from the step 10 in to an already prepared Zymo-Spin IV- HRC Spin Filter (green top) in a clean new collection tube and centrifuged at 8000×g for 1minute. The filtered DNA was collected into a 500µl centrifuge tube and stored at -20˚C.

APPENDIX IV

CALCULATION FORMULA FOR THE PURIFICATION PROFILE

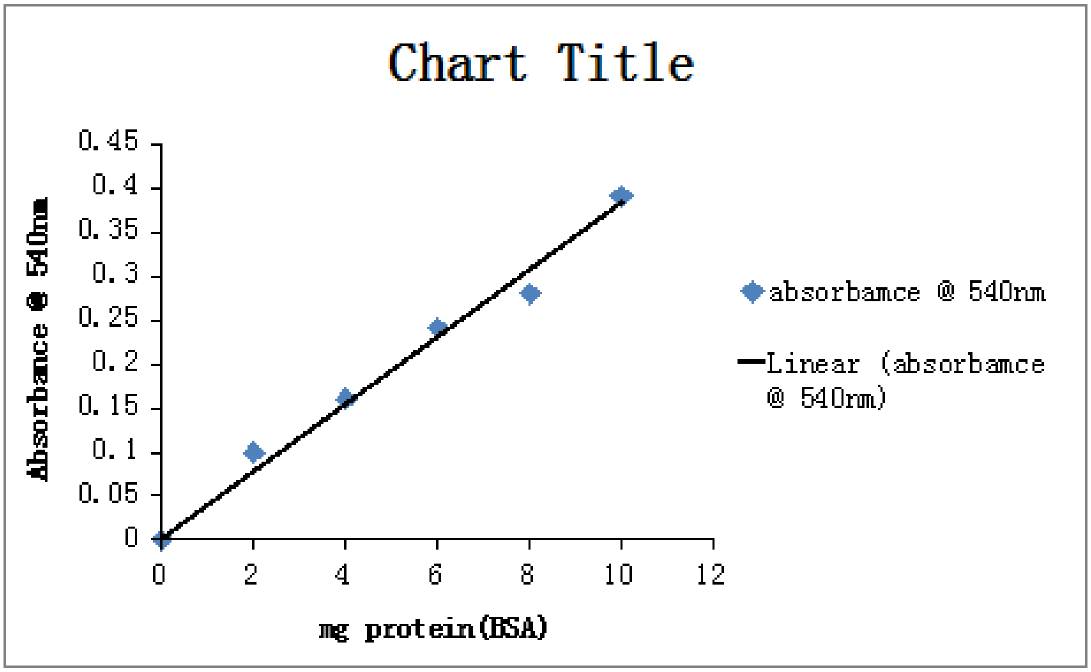
$$\text{Specific Activity} = \frac{\text{Total Activity}}{\text{Total Protein}}$$

$$\text{Purification Fold} = \frac{\text{Specific Activity of Protein at a Purification Step}}{\text{Original Specific Activity (Crude)}}$$

$$\text{Percentage Yield} = \frac{\text{Total Activity of protein at a step}}{\text{Total Activity of Original Purification (Crude)}} * 100$$

APPENDIX V

STANDARD CURVE FOR PROTEIN ESTIMATION USING BSA



APPENDIX VI

CALCULATIONS FOR DNA CONCENTRATION AND QUALITY.

The following formula was used in the calculation of the quality and concentration of the DNA isolated with a dilution factor of 10.

$$\text{Adequate purity} = \frac{A260nm}{A280nm} = 1.7 - 2.0$$

$$\text{Concentration of DNA } (\mu g/\mu l) = \frac{A260 \times 50 \mu g/ml \times \text{dilution factor}}{1000} = X \mu g/\mu l.$$

The following readings and results were obtained:

PLANTS	A260nm	A280nm	A260/A280 (QUALITY)	CONCENTRATION
Albizia lebbeck	0.4330	0.2456	1.763	0.2165µg/µl
Piliostigma thonningii	0.3820	0.2070	1.840	0.1910µg/µl