ISOLATION AND PARTIAL PURIFICATION OF LECTINS FROM THE STEM BARK OF ORDEAL TREE (*Erythrophleum suaveolens*) AND THEIR POSSIBLE LARVICIDAL ACTIVITIES AGAINST *Culex quinquefasciatus*

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DEPARTMENT OF BIOCHEMISTRY
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ZARIA

FEBRUARY, 2013

DECLARATION

I hereby declare that this thesis entitled "Isolation and Partial Purification of Lectins from the Stem Bark of Ordeal Tree (*Erythrophleum suaveolens*) and their Possible Larvicidal Activities against *Culex quinquefasciatus"* is a record of my own research work under the supervision of Dr. H.M. Inuwa and Dr. S. Ibrahim. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project has been previously presented elsewhere for the award of a degree in any other university.

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Name of Student	Signature	Date

CERTIFICATION

This project titled "Isolation and Partial Purification of Lectins from the Stem Bark of Ordeal Tree (*Erythrophleum suaveolens*) and their Possible Larvicidal Activities against *Culex quinquefasciatus*" meets the regulations governing the award of the degree of Master in Science of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

Dr. H.M. Inuwa Chairman, Supervisory Committee	_____ Signature	_____ Date
Dr. S. Ibrahim Member, Supervisory Committee	_____ Signature	_____ Date
Dr. H.M. Inuwa Head of Department	_____ Signature	_____ Date
Prof. A. A. Joshua Dean, School of Postgraduates Studies	_____ Signature	_____ Date

DEDICATION

This research work is dedicated to my beloved father, Dr. A.A. Ihuoma.

ACKNOWLEDGEMENT

I profoundly thank God Almighty who made all things possible, for His Wisdom, Knowledge, Love, Faithfulness and Inspirations to carry out this research work.

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ABSTRACT

Lectins from the saline extract of *Erythrophleum suaveolens* stem bark, were isolated, partially purified, characterized and evaluated in terms of their larvicidal properties against *Culex quinquefasciatus.* Purification was done by precipitation with ammonium sulfate at 20% - 90% saturation. The ammonium sulfate precipitate was subjected to gel filtration using Sephadex G-75 resin. The two fractions with the highest heamaglutinating activity were further subjected separately to ion-exchange chromatography using Sp-Sephadex C-50 resin. The lectins in the crude extract and the purified lectins appeared as five bands at positions 38KDa, 28KDa, 26KDa, 11KDa and 9KDa on a SDS-PAGE electrophoregram. The lectins were found to be stable at the temperature range of 30-60^0C and pH range of 3-7. The carbohydrate content of the crude extract, Gel I, Gel II, Ion I and Ion II peaks were 0.04%, 0.04%, 0.03%, 0.02%, and 0.01% respectively and showed specificity to mannose and galactose. A series of nine concentrations of the extracts ranging from 0.0001 mg/L, 0.001 mg/L, 0.01 mg/L, 0.1 mg/L, 1mg/L, 5mg/L, 10 mg/L, 50 mg/L and 100mg/L were tested against the third instar larvae of C*ulex quinquefasciatus* and their percentage mortalities and LC_{50} values were obtained. The crude extract of *Erythrophleum suaveolens* show very high larvicidal activity with 96% mortality achieved at a concentration of 100 mg/L and had an LC_{50} of 4.39 mg/L. The gel peaks of *Erythrophleum suaveolens* showed larvicidal activities to C*ulex quinquefasciatus* larvae with 74.67% and 69.33% mortality achieved at a concentration of 100 mg/L and had LC_{50} of 43.73mg/L and 43.65mg/L respectively. The ion exchange purified extracts of *Erythrophleum suaveolens* showed low larvicidal activity to C*ulex quinquefasciatus* larvae with 10.67% and 9.33% mortality achieved at a concentration of 100 mg/L respectively. These results suggest that the saline stem bark extract of *Erythrophleum suaveolens* is promising as a larvicide against mosquito larvae and can be used directly in small volumes in aquatic habitats and in mosquito population management programme.

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LIST OF ABBREVIATIONS

PBS	-	Phosphate Buffered Saline
BSA	-	Bovine Serum Albumin
APS	-	Ammonium per Sulphate
TEMED	-	Tetramethylethylenediamine
HA	-	Haemagglution Assay
HU	-	Haemagglution Unit
AOAC	-	Association of Official Analytical Chemists
SDS-PAGE	-	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
ANOVA	-	Analysis of Variance

CHAPTER ONE

INTRODUCTION

1.1 GENERAL INTRODUCTION

Insect transmitted diseases are important health problems in the world. These vectors are insects such as mosquitoes (Roberts, 2002). Mosquitoes are among the most important group of insects in terms of their medical importance to both humans and animals. Most insecticides are non-selective, not biodegradable and can be harmful to other organisms and to the environment. An approach to obtain new efficient, safe and selective insecticides is the study of natural models such as the defensive mechanisms of plants (Ciccia *et al.,* 2000). Bioactive organic compounds produced by plants can act as repellant, food deterrents, growth inhibitors, and toxins (Ezeonu *et al.,* 2001; Carlini and Grossi-de-Sá, 2002). Thus, crude plant extracts have been screened as natural and biodegradable forms to control pests and vectors of infectious diseases (Omena *et al.,* 2007). Plant essential oils are, in some cases, highly active and economically viable for Insect control (Silva *et al.,* 2008). The larvicidal activity of essential oils from *Hyptis fruticosa, Hyptis pectinata* and *Lippia gracilis* has been reported (Silva *et al.,* 2008). Lectins, ribosome-inactivating proteins (RIPs), inhibitors of enzymes, glycohydrolases, arcelins, chitinases, canatoxins and modified forms of storage proteins are examples of proteins that are associated with defensive mechanisms of plants. All these protein classes have been shown to have entomotoxic activities on Coleoptera and some of them demonstrated toxic effect on other insect orders (Carlini and Grossi-de-Sá, 2002).

1.2 LECTINS

Lectins are glyco-proteins of non immune origin that agglutinate cells or precipitate complex carbohydrates. It is their unique ability to recognize and bind reversibly to carbohydrate ligands without inducing chemical modifications in their covalent structure

that distinguishes them from other carbohydrate binding proteins. Lectins exhibit high levels of specificity and affinity towards the sugar moieties of the glyco-conjugates, but do not possess enzymatic activity (Peumans and Van Damme, 1995; Gabius *et al*., 2002; Loris, 2002). The interaction between a lectin and a sugar moiety occurs at the carbohydrate-recognition domain and involves Vander Waals forces and the formation of hydrogen bonds between sugar hydroxyl groups and amino acid residues at the active site of the lectin (Weis and Drickamer, 1996). Lectins typically possess shallow binding pockets that are solvent-exposed; therefore, the interaction between the carbohydrate and the protein is typically weak (Atul *et al.,* 2007). To provide interaction strength and specificity, many carbohydrate binding proteins are oligomeric, consisting of several similar or identical monomers that bind to carbohydrate each (Atul *et al.,* 2007).

The cross-linkages formed between adjacent cells through binding between lectins glycol-conjugates causes cell agglutination (Peumans and Van Damme, 1995). When erythrocytes are involved in this type of interaction, the phenomenon of haemagglutination occurs (Alonso *et al.,* 2001). Ingestion of lectins by animals may cause anti-nutritional effects, degeneration of cell membranes and inhibition of digestive enzymes (Vasconcelos and Oliveira, 2004) and the resulting interference with the absorption of nutrients may give rise to serious physiological consequences. On the other hand, the strong sugar specificity of lectins has led to their exploitation in many biological and medicinal areas, and they have found application as fungicidal, bactericidal and insecticidal agents in agriculture (Peumans *et al*., 2000; Grahamstown and Van Staden, 2002), in the purification and characterisation of polysaccharides and in the glycol-conjugates industry (Lima *et al.*, 1997). Furthermore, it is used in the identification of blood groups (Lis and Sharon, 1998), as stimulators of lymphocyte mitogenesis and immunological functions (Sharon and Lis, 2004), in the agglutination of

cancer cells and in studies of oncogenesis (Sharon and Lis, 2004) and in the investigations concerning the structures of proteins and carbohydrates in cells (Silva and Silva, 2000**).** Lectins play an important role in plants defense against insect pests, and have been found to be toxic to viruses, bacteria, fungi, insects and higher animals (Sauvion *et al*., 2004). The lectin binding to carbohydrates of plasmatic membranes promotes erythrocyte network resulting in the agglutinating phenomenon (Leite *et al.,* 2005). This carbohydrate recognition property is involved in the lectin entomotoxic activity on larvae, developing stages and mature forms of insects (Bandyopadhyay *et al.,* 2001; Macedo *et al.,* 2002; Macedo *et al.,* 2004; Sauvion *et al*., 2004; Leite *et al.,* 2005; Kaur *et al.,* 2006). Lectins can be explored for insect control or by genetic engineering aiming to increase resistance of plants to insects (Carlini and Grossi-de-Sá, 2002; Saha *et al.,* 2006). The midgut of insects contains a membrane, the peritrophic matrix, which separates the contents of the gut lumen from the digestive epithelial cells lining the midgut. It has been reported that chitin and glycosylated proteins containing –N-acetylglucosamine of peritrophic matrix are the targets for lectin binding (Tellam *et al.,* 1999).

The lectin, with chitin-binding property, isolated from *Annona coriacea* seeds was reported to promote larval mortality of flour moth *Anagasta kuehniella*. The larvicidal activity was related to lectin binding to chitin components in the insect gut, interaction with glycoconjugates on the surface of epithelial cells along the digestive tract, binding to the sugar moiety of glycosylated digestive enzymes or assimilatory proteins and resistance to insect digestive proteases (Coelho *et al.,* 2007). Bruchid beetle larvae of *Zabrotes subfasciatus* and *Callosobruchus maculatus* were sensitive to *Bauhinia monandra* leaf lectin that, although belongs to the galactose group of specificity, was able to bind to chitin, a polymer of -N-acetylglucosamine (Macedo *et al*., 2007).

1.3 *Erythrophleum suaveolens*

Erythrophleum suaveolens commonly called "ordeal tree" is known as *Erun obo* in Yoruba, *Gwaska* in Hausa and *Inyi* in Ibo (Lawal, 2010). It is a poisonous plant that is widespread in tropical Africa, belonging to the *Fabaceae* family. It is a large tree with a smooth bark which is pale grey, with large irregularly shaped thin pieces peeling off the lower trunk. The leaves are 8 to 20 alternate and terminal leaflets (Burkill, 1995).

1.4 THE MOSQUITO VECTOR

Mosquitoes are flying, biting insects that develop in water during their immature stages. Some of the many species found in the world are considered pests and can transmit diseases to humans. The three most important mosquito groups are the *Anopheles* (carrier of malaria), *Culex* (carrier of viral encephalitis), and *Aedes* (carrier of yellow fever, dengue, and encephalitis) (Anonymous, 2009). With the constantly changing environment, the epidemiology of mosquito-borne diseases is also modified. Global warming, periodic flooding and deforestation have opened new habitats to mosquitoes which show high plasticity in their breeding behavior and readily spread their distribution (Anonymous, 2009). Although mosquitoes are usually a nuisance and sometimes dangerous to public health, most specialists agree that complete eradication is unrealistic. A more reasonable goal is population reduction and management below problem level (Anonymous, 2009). All mosquitoes must have water in which to complete their life cycle. This water can range in quality from melted snow water to sewage effluent and it can be in any container imaginable (Anonymous, 2009). The type of water in which the mosquito larvae is found can be an aid to the identification of which species it may be (Anonymous, 2009). The adult mosquitoes show a very distinct preference for the types of sources in which to lay their eggs (Anonymous, 2009). They lay their eggs in such places such as tree holes that periodically hold water, tide water pools in salt marshes,

sewage effluent ponds, irrigated pastures, rain water ponds and so on. Each species therefore has unique environmental requirements for the maintenance of its life cycle (www.mosquitoes.org/LifeCycle.html 1983).

Biological control generally, involves using living organisms to control a specific pest. When a predator, parasite, or plant extract is chosen to attack a harmful insect, nature is manipulated to achieve a desired effect. As part of an overall Integrated Pest Management (IPM) program, biological control methods can reduce the legal, environmental, low water quality and health hazards of using chemicals in the environment, thereby preventing economic damage to the plants and animals. These control methods remain in effect year after year, limiting pests without any additional costs or synthetic additives to the natural environment (Anoynmous, 2013).

1.5 JUSTIFICATION

The use of synthetic insecticides in the control of insects is expensive (Franzen, 1993). There are problems of pathogen resistance and negative effects on non-target organisms including humans and the environment in terms of pollution (F.A.O.1992, Franzen, 1993, Rembold, 1984). There is a need, therefore, to identify botanical insecticides which are toxic to insects but at the same time do not exhibit toxicity to mammals and humans (Jbilou *et al*., 2006). These would be useful in crop protection applications without restriction on the food use of the material in which the foreign lectin is to be presented**.** Higher plants are a rich source of novel natural substances that can be used to develop environmental safe methods for insect control (Jbilou *et al.,* 2006). The application of natural insecticides and fungicides could provide a cheap and environmentally friendly alternative (Macedo *et al*., 2000). Therefore, there is a need to curtail the presence of chemicals in the environment as a means of guarding against their adverse effect on the environment, by employing environmentally safe method of insect and pest control, using

lectins from *Erythrophleum suaveolens.* The wood of *Erythrophleum suaveolens* is resistant to termite and fungi biodegradation (Okeyo, 2006). Although this information has been available for hundreds of years and hence the popularity of *Erythrophleum suaveolens* for construction purposes, there is no scientific investigation to determine the toxic activity against insects or the mode of action of the active substances. Based on the foregoing, this study sort to scientifically evaluate the larvicidal activity of the *Erythrophleum suaveolens* on the *Culex quinquefasciatus* larvae and its mode of action and application.

1.6 GENERAL AIM

The aim of the this study is to isolate, partially purify and characterize the lectins from *Erythrophleum suaveolens* bark in order to evaluate their larvicidal activity on the larvae of *Culex quinquefasciatus*.

1.7 SPECIFIC OBJECTIVES

1. Isolate lectin from the stem bark of *Erythrophleum suaveolens*.
2. Partially purify and characterize the lectin from the stem bark of *Erythrophleum suaveolens.*
3. Investigate the larvicidal potency of *Erythrophleum suaveolens* lectin on the third instar larvae of *Culex quinquefasciatus.*

CHAPTER TWO

LITERATURE REVIEW

2.1 THE PLANT

2.1.1 Plant Description

Erythrophleum suaveolens, a western African tree, consists of about 10 species that are mainly native to Tropical Africa (Dongmo *et al.*, 2001). The British colonialists referred to *Erythrophleum suaveolens* as red water tree (Adeoye and Oyedapo, 2004). *Erythrophleum suaveolens* is a medium-sized tree up to 25–30m tall, often branching low; the bark is finely fissured, scaly, and grey. The leaves alternate between 7–14 per pinna and inflorescences an axillary panicle consisting of spike-like racemes up to 12 cm long which are short yellowish and hairy. The flowers are bisexual, regular, yellowish white to greenish yellow with calyx lobes of about 1–1.5 mm long; petals 2–3 mm × 0.5 mm, which are short and hairy at margins. The fruit is flat, slightly curved, with dehiscent pod 5–17 cm × 3–5 cm, the stripe is often lateral, broadly rounded at apex and pendulous, contains about 6 -11 seeds. The seeds are oblong-ellipsoid in shape. The main parts of *Erythrophleum suaveolens* harvested for medicinal purposes are the roots and bark, while the wood is exploited for timber. Traditional herbalists use ad hoc techniques to excavate the roots and de-bark the stems (Okeyo, 2006). Virtually all trees are used, from the young to the old ones (Okeyo, 2006).

2.1.2 Taxonomic Hierarchy

Kingdom: *Plantae*

Subkingdom: *Tracheobionta*

Division: *Magnoliophyta*

Class: *Magnoliopsida*

Subclass: *Rosidae*

Order: *Fabales*

Family: *Fabaceae*

Genus: *Erythrophleum*

Species: *Erythrophleum suaveolens* (Anonymous, 2007).

2.1.3 Properties of *Erythrophleum suaveolens*

Erythrophleum suaveolens bark is rich in alkaloids which ranges from 0.3% to 1.5% and varies with the age of the tree (Cronlund, 1976): an extract of the bark contained 0.5% and 0.9% respectively in a 60 year old and 150 year tree (Cronlund, 1976). The alkaloids are esters of tricyclic diterpene acids, and 2 main types exist: dimethylaminoethylesters and monomethylaminoethylesters (nor-alkaloids). The bark contains as main components alkaloids of the dimethylaminoethylester type: cassaine, erythrophlamine, erythrophleguine and norcassamidine as well as the amide erythrophlamide (Cronlund, 1976). The bark extract has an excellent local anaesthetic activity on the eyes and skin, although it causes irritation on the conjunctiva, trials using it as anaesthetic in operations or tooth extractions were less successful, because symptoms of poisoning occured in higher doses (Neuwinger,1996). It acts as a strong, rapid-acting cardiac poison, in warm-blooded animals causing shortness of breath, seizures and cardiac arrest in a few minutes (Abbiw, 1996).). The alkaloids also have strong diuretic effects, and increase contractions of the intestine and uterus (Naderali *et al.,* 2000). Apart from an increase of heart

contraction in systole, the alkaloids also demonstrated an increase in diastole (Kamanyi *et al.,* 2003). In addition, cassaine caused a violent state of excitation. Although the alkaloid content in the seeds is markedly lower than in the stem bark, the seeds are more toxic (Cronlund, 1976). This strong activity is due to a strong haemolytic saponin, which acts in a synergistic way to the alkaloids. Norcassamidine has local anaesthetic action, and is a convulsing agent (Mgbenka, 1998). The bark further contains procyanidins (polyphenols) and hence has antioxidant properties. The relaxant effect of the bark extract of *Erythrophleum suaveolens* is due to its procyanidins (Kamanyi *et al.,* 2003). The bark also contains the trihydroxystilbene resveratrol. Resveratrol shows antiplatelet aggregation, coronary vasodilator, antileukaemic, antifungal and protein-tyrosine kinase inhibitory activities (McGaw, 1997). Trihydroxystilbenes are thought to protect against atherosclerosis and coronary heart disease (Marshall, 1998). The wood is durable and resistant to fungi, dry wood borers and termites (Okeyo, 2006).The sawdust may irritate mucous membranes and may cause allergy and asthma of labourers in sawmills (Okeyo, 2006).

2.1.4 Distribution of *Erythrophleum suaveolens*

Erythrophleum suaveolens is widespread and locally common West Africa, and is as such not threatened by genetic erosion, occurs in moist semi-deciduous forests, gallery forest and wooded grasslands, from sea-level up to 1100 m altitude. It is absent from the evergreen forest (Burkill, 1995). It can be propagated in nurseries and seed takes three weeks to germinate (Okeyo, 2006). In Nigeria, *Erythrophleum suaveolens* is cultivated in the south-eastern states and eaten mainly by the Ibos (Adedotun *et al.,* 2006).

2.1.5 Medicinal Uses of *Erythrophleum suaveolens*

Erythrophleum suaveolens is used as emetic and purgative (Burkill, 1995). The crushed bark is applied to swellings caused by *Filaria* (Oliver-Bever, 1986). In Congo the dried

powdered bark is taken as a snuff to cure headache (Okeyo, 2006). In Kenya a diluted decoction of the roots is used as an anthelminthic, especially against tapeworm (Beentje, 1994). In Malawi a decoction of the roots and bark is applied to soothe general body pain (Okeyo, 2006). In the Western world, bark extracts were used in the late 19th century to treat heart failure. Side effects and better results with digitoxine ended this practice (Okeyo, 2006).

2.1.6 Economic Uses of *Erythrophleum suaveolens*

Traditionally the plant is used as an ordeal poisons to determine innocence or guilt for witches (Robert and Wink, 1998). The bark has been used in arrow poisons as an anaesthetic for fish in aqua-culture, it is tricky as small differences in dosage will kill, rather than stupefy the fish. The tree produces quite hard, heavy and durable wood, which is suitable for joinery, flooring, railway sleepers, harbour and dock work, turnery, construction of bridges, boat building and wheel hubs. The bark has been used in West Africa for tanning hides and skins; it is used in Congo to colour leather brown (Okeyo, 2006). In West Africa the powdered bark is mixed with the residue of palm oil processing, and after boiling it is mixed with seeds of maize, cowpea or cotton, which effectively reduces pest damage to the seeds. Dried leaves are mixed with stored grains and pulses to repel or kill storage insects (Okeyo, 2006). Its use in agro-forestry promotes nitrogen fixation and the large amount of leaf litter is advantageous to intercrops (Sprent, 2005).

2.1.7 Nutritional Uses of *Erythrophleum suaveolens*

The seeds of *Erythrophleum suaveolens* are relatively cheap to purchase and have replaced melon “egusi” in the south-eastern Nigeria (Adedotun *et al*, 2006). In Uganda, the seed is a favourite food of elephants and they are reported to be responsible for dispersing the seeds (Burkill, 1995).

2.2 LECTINS

To cope with the continuous threat from different phytophagous insects, plants have evolved a whole arsenal of defense strategies (Michiels *et al.,* 2010). These defense mechanisms include morphological andstructural features as well as the synthesis of chemical compounds. Chemical defense products may range from low molecular weight compounds called secondary metabolites to peptides and proteins that are active against pest insects (Vandenborre *et al.,* 2009b). One particular group of such defense proteins is called plant lectins or plant agglutinins (Van Damme, 2008; Vandenborre *et al.,* 2009b; Michiels *et al.,* 2010). Plant lectins are a very heterogeneous group of proteins that all share one important biological property: they can recognize and bind reversibly to specific carbohydrate structures (Van Damme, 2008). Analyses at the level of the genome and the transcriptome have shown that lectin sequences are ubiquitous in the plant kingdom and hundreds of lectins have already been purified from various plant species (Van Damme *et al.,* 2008).

The first discovery of a plant lectin dates back to the end of the nineteenth century in the year 1888, ricin was found castor bean (*Ricinus communis*) by Peter Hermann Stillmark (Van Damme *et al.,* 2008). Since ricin was shown to agglutinate red blood cells, the term hemagglutinin was introduced. Later, it was recognized that some hemagglutinins can selectively agglutinate human erythrocytes depending on their ABO blood group type. This finding gave birth to the word “lectin” which is derived from “legere”, the Latin verb for “to select” (Van Damme *et al.,* 2008). The selectivity of plant lectins is based on the capacity to bind different carbohydrate structures present on the cell membrane of erythrocytes (Peumans and Van Damme, 1995). The term plant lectin is used to denote all plant proteins possessing at least one non-catalytic domain that binds reversibly to a specific mono- or oligosaccharide (Peumans and Van Damme, 1995).

2.2.1 Classification of Plant Lectins

Based on the overall domain architecture of plant lectins, four major groups can be distinguished: merolectins, hololectins, chimerolectins and superlectins (Van Damme *et al.*, 1998a).

Merolectins are proteins that contain only one carbohydrate-binding domain. Due to their monovalent nature, this group of lectins cannot agglutinate cells (Van Damme *et al.,* 2008). Hololectins are lectins that are composed of two or more identical or very homologous carbohydrate binding domains which allow them to agglutinate cells and/or precipitate glycoconjugates (Van Damme *et al.,* 2008). Most of the currently isolated and characterized plant lectins belong to the group of hololectins. In contrast to this lectin group, superlectins are composed of at least two carbohydrate-binding domains that recognize structurally unrelated carbohydrate structures (Van Damme *et al.,* 2008). The group of chimerolectins comprises all plant lectins that are composed of one or more carbohydrate binding domains fused to a domain that exerts a biological activity independent from the carbohydrate binding domain (Van Damme *et al.,* 2008). Sequence analyses of complete plant genomes revealed that chimerolectins are very abundant in plants (Van Damme *et al.,* 2008). Moreover, recent genome/transcriptome analyses of plants provided evidence for the occurrence of many proteins containing one or more lectin domain embedded in a more or less complex multi-domain architecture (Van Damme *et al.*, 2008). Because of this complexity and heterogeneity among all carbohydrate binding proteins, it is preferable to consider the class of plant lectins in terms of the carbohydrate-binding domains present in these proteins (Van Damme *et al.,* 2008).

2.2.2 Types of Lectins

2.2.2.1 Legume lectins

These are lectins that have similar properties to the C-type lectins. Concavalin A is a legume lectin which has two metallic binding sites. However, the calcium cation occupies only one site, where as manganese binds to a secondary site. The binding of these two metallic atoms occurs in a sequential order (Thiel *et al*., 1997). Calcium is added to the protein first, which changes the protein structure and allows the uptake of manganese. When both sites are filled the protein structure is available for saccharide binding (Thiel *et al*., 1997). In addition, it has been observed that using secondary cations such as cadmium or zinc in place of calcium and manganese does not change the proteins carbohydrate binding affinity or the lectin's biological role (Thiel *et al*., 1997). Therefore it can be concluded that for C-type lectins and several legume lectins, the presence of metallic cations are necessary for functionality (Thiel *et al*., 1997). Another factor, contributing to the regulation of lectins is the pH of the system, lowering the pH below 6.5 gives rise to conformational changes that lead to the inactivation of lectin proteins (Drickamer, 1988). Although, this information explains the regulation of the lectins on a molecular level, it is also worthy of note to mention their genetic regulatory properties. Since lectins are proteins that have functions in biological systems as "decoders" of information, it follows that the cell contains information for their production. Several lectins have been found to be linked to specific genes (Drickamer *et al.,* 1987). In particular, mannose-binding protein, a lectin that has a high affinity for mannose, has had its genetic sequence mapped. It has been found to be regulated by a gene containing three exons -structural genes and two introns–non structural genes which are spliced out during post translational modification (Drickamer *et al.,* 1987).

2.2.2.2 Chitin-Binding Lectins

Chitin-binding lectins include all proteins with at least one domain which is structurally and functionally related to hevein. Hevein refers to a small chitin-binding protein from the latex of the rubber tree (*Hevea brasiliensis*). Chitin-binding lectins are widespread and occur in seeds and vegetative tissues of monocots and dicots. Concentrations vary from very low (Gramineae seed lectins: 0.02% of total seed protein) to very high (*Datura* lectin: 50% of total seed protein).The seeds of many species of the Gramineae contain lectins, which specifically bind the sugar N-acetylglucosamine (GlcNAc), its oligomers, and chitin, a polymer of GlcNAc residuesIn hexaploid wheat, the GlcNAc binding lectin Wheat germ aggluthin (WGA) consists of three unique isolectins (A, B, and D) encoded by homologous genes within the respective diploid genomes. The cDNA clones encoding isolectins A, B, and D show 93% to 95% identity at the amino acid level (Wright and Raikhel, 1989) and at the nucleotide level (Raikhel and Wilkins, 1987; Smith and Raikhel, 1989a). Lectins very similar to WGA by immunological, biochemical, and sugar binding criteria are also present in rye (*Secale cereale*) (Peumans *et al.,* 1982a), barley (*Hordeum vulgare*) (Peumans *et al.,* 1982a), rice (*Oryza safiva*) (Tsuda, 1979), couch grass (*Agropyrum repens*) (Cammue *et al.,* 1985), and false brome grass (*Brachypodium sylvaficum*) (Peumans *et al.,* 1982b). Gramineae lectins are synthesized as preproproteins; their hydrophobic signal peptides are cotranslationally removed and they are modified by the addition of a high mannose glycan on the carboxyl-terminal propeptide (Mansfield *et al.,* 1988; Smith and Raikhel, 1989b).

2.2.2.3 Monocot Mannose-Binding Lectins

Monocot mannose-binding lectins have exclusive recognition of mannose and manno-oligosaccharides. The first member of this family was the lectin isolated from snowdrop (*Galanthus nivalis*) bulbs, a member of the Amaryllidaceae family (Van Damme *et al*.,

1987). By means of biochemical analysis and molecular cloning, identification and characterization of related lectins from other member families, namely, Alliaceae, Araceae, Bromeliaceae, Orchidaceae, Liliaceae and Zridnceae have been identified (Barre *et al*., 1996; Van Damme *et al*., 2000). Other than unique specificity for mannose, these lectins have re-semblance with respect to their amino acid composition, sequence and molecular structure.

Currently, there is a wide interest in the monocot mannose-binding lectins because of their exclusive specificity towards mannose, potent inhibitory effect on animal and human retro-viruses, ability to block the adhesion receptors of mannose-fimbriated *Escherichia coli* in the small intestine of rats, use in the analysis and isolation of mannose containing glycoconjugates and use in plant protection as their genes confer resistance against insects and nematodes. Their strict specificity for mannose sets them apart from the Glc/Man/Gal specific family of dicotyledonous legume lectins and the C-type mannose binding animal lectins (Van Damme *et al*., 2000).

2.2.2.4 Type-2 Ribosome Inactivating Proteins

Many plants contain one or more so-called ribosome-inactivating proteins (RIP). RIP are *N*-glycosidases that catalytically inactivate eukaryotic ribosomes (Barbieri *et al.,* 1993) by removing a single adenine residue from the large rRNA. RIP are divided into three groups on the basis of their molecular structure. Type 2 RIP are built up of one or more protomers consisting of two different disulfide-linked A and B chains. The A chain of the [A-s-s-B] pair shows sequence similarity to type 1 RIP and has RNA *N*-glycosidase activity, whereas the B chain is devoid of enzymatic activity, but contains carbohydrate-binding sites. Due to the presence of these carbohydrate-binding sites, type 2 RIP are also regarded as lectins (Peumans and Van Damme, 1995). Hitherto, type 2 RIP have been identified in *Abrus precatorius* (Fabaceae), *Adenia digitata* and *A. volkensii*

(Passifloraceae), castor bean (Euphorbiaceae), *Viscum album* (Viscaceae), *Cinnamonum camphora* (Lauraceae), *Eranthis hyemalis*(Ranunculaceae), and *Sambucus nigra*, *Sambucus ebulus*, and *Sambucus sieboldiana* (Sambucaceae;Van Damme *et al.,* 1998). It has been claimed that seeds of the camphor tree (Ling *et al.,* 1995) and the leaves/bark of dwarf elder (Girbes *et al.,* 1993; Benito *et al.,* 1995) and elderberry (Benito *et al.,* 1998) express simultaneously type 1 and type 2 RIP. All type 2 RIP characterized hitherto contain binding sites that accommodate Gal or *N*-acetylgalactosamine (or both) except elderberry agglutinin I, which harbors a Neu5Ac (α, 2–6) Gal/GalNAc-binding site (Van Damme *et al.,* 1996). There are good indications from structural studies of other plant lectin families that even fairly well-conserved binding sites can exhibit a different specificity, it is questionable whether the specificity of type 2 RIP is really restricted to Gal, *N*-acetylgalactosamine, and Neu5Ac(α, 2–6) Gal/GalNAc (Van Damme *et al.,* 1996).

2.2.3 Structure of Plant Lectin

Most of the leguminous plant lectins contain at least one N-glycan, but some lectins, such as Concavalin A, do not contain any covalently associated carbohydrate (Loris *et al.,* 1998). The ease of purification and availability have made plant lectins prime subjects of study for protein crystallography, and the structures of many plant lectins have now been deduced (Wright, 1992). For the most part, leguminous lectins assemble into a compact β-barrel configuration devoid of α-helices and dominated by two antiparallel pleated sheets. In leguminous lectins, the metal-binding sites are located on a single long loop (Bourne *et al.,* 1994). The binding site for carbohydrate in most leguminous lectins involves a combination of hydrogen bonds, hydrophobic interactions, and van der Waals contacts (Rüdiger, 1997). The metals near the binding site do not make direct contact with the sugar but help stabilize amino acid side chains required for binding. The plant

lectins appear to acquire high affinity via the multivalency they display in dimeric and tetrameric forms (Rüdiger, 1997). The quaternary structure of the lectins may also contribute to glycan recognition. The ability of each subunit to individually bind sugars can lead to unique cross-linked lattice structures, as have been observed exquisitely for soybean agglutinin in its interactions with four isomeric biantennary N-glycans and for wheat-germ agglutinin (Olens *et al.,* 1997). This ability to form lattice structures allows lectins to form complex interactions with cell surfaces and matrix glycoconjugates containing multiple binding sites, which probably contributes to the biological activities of plant lectins (Loris *et al.,* 1996).

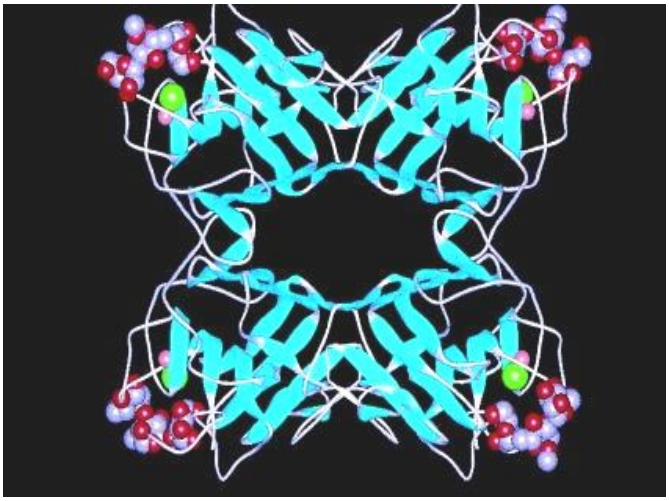


Figure 2.1: Structure of Tetrameric Concavalin A at 2.35 Å (Naismith, *et al.*, 1996).

2.2.4 Mechanism of Action of Plant Lectins

2.2.4.1 Target Structures or Tissues for Plant Lectins in the Insect Body

The luminal side of the midgut epithelium of many insects is lined up with a physical barrier called the peritrophic membrane that is secreted by certain epithelium cells (Hegedus *et al.,* 2009). This peritrophic membrane consists of a chitineous grid-like network held together by chitin-binding glycoproteins such as peritrophins. The peritrophic membrane - associated glycoproteins contain many glycan structures that fill the interstitial spaces creating a molecular sieve (Hegedus *et al.,* 2009). Since both the chitin fibrils and many glycoproteins are present in the peritrophic membrane, this midgut structure is an obvious target for lectins. Studies have shown clear abnormalities in the formation of a functional peritrophic membrane and disruption of microvilli structures. Analysis of the midgut structures in the European corn borer (*Ostrinia nubilalis*) after feeding on a wheat germ agglutin (WGA) containing diet showed hypersecretion of many disorganized peritrophic membrane layers into the midgut lumen and the presence of many disintegrated microvilli (Harper *et al.,* 1998; Hopkins and Harper, 2001).

2.2.4.2 Insect Glycoproteins as Targets for Lectins

Binding studies using glycan arrays have shown that many plant lectins have a strong affinity for glycans that are frequently present on insect proteins. Because most of the digestive enzymes or transport proteins secreted in the midgut of insects or proteins embedded in the epithelial cell membrane contain such glycan structures, these glycoproteins are all potential targets for plant lectins. A major target protein for Galanthus nivalis agglutinin (GNA) was shown to be a subunit of ferritin, an important protein for iron transport (Du *et al.,* 2000; Sadeghi *et al.,* 2008b). An enzyme aminopeptidase was the target of GNA and ConA in the midgut of the pea aphid

(*Acyrthosiphon pisum*) (Cristofoletti *et al.,* 2006). Several plant lectins were also shown to bind to a-amylases present in the digestive tract (Macedo *et al.,* 2007; Sadeghi *et al.,* 2008b). The presence of target proteins for GNA in Drosophila illustrated that plant lectins probably act on insects through the interaction with multiple target glycoproteins (Vandenborre *et al.,* 2010b). This was also shown for pest insects such as beetles, aphids and caterpillars, and for non-target insects such as honeybees (Vandenborre *et al.,* 2011). Secreted glycoproteins targeted by plant lectins in the midgut tend to cluster, resulting in large complexes with a molecular size that is too big to pass through the peritrophic membrane and may prevent larval enzymes from diffusing back across the peritrophic membrane for being recycled in the digestive system and will result in leakage of digestive enzymes (Vandenborre *et al.,* 2011).

2.2.4.3 Effect of Plant Lectins on Gene Expression

To study the observed structural changes of the insect midgut microvilli after uptake of dietary plant lectins in more detail, the differential expression of genes was analyzed in epithelium cells of *Drosophila melanogaster* larvae after feeding on a wheat germ agglutinin (WGA) containing diet (Li *et al.,* 2009). Microarray analysis revealed that 61 transcripts were differentially expressed in midgut cells after feeding of Drosophila larvae on a wheat germ agglutinin (WGA) rich diet (Li *et al.,* 2009). The genes with altered expression levels in response to wheat germ agglutinin (WGA) uptake were associated with cytoskeleton organization, chitin metabolism, digestive enzymes, detoxification reactions and energy metabolism (Li *et al.,* 2009). Differential expression of genes involved in cytoskeleton organization is in agreement with the morphological changes in micro-villi after wheat germ agglutinin (WGA) ingestion (Li *et al.,* 2009).

2.2.5 Applications of Lectins

2.2.5.1 Plant defense

Molecular, biochemical, cellular, physiological and evolutionary arguments indicate that lectins have a role in plant defense (Van Damme *et al*., 1994). This is primarily based on the fact that lectins bind to glycoconjugates of other organisms (Van Damme *et al*., 1994). Although many plant lectins are able to bind simple sugars such as glucose, mannose or galactose, they have a much higher affinity for oligosaccharides, which are not common or totally absent in plants (Van Damme *et al*., 1994). For instance, chitin binding plant lectins recognize a carbohydrate that is a typical constituent of the cell wall of fungi and the exoskeleton of invertebrates (Van Damme *et al*., 1994). A circumstantial argument in favor of a defense role of plant lectins is their marked stability under a wide range of pH, heat and exposure to proteases (Van Damme *et al*., 1994). The preferential association of lectins with those parts of the plant such as resting storage organs and seeds that are most susceptible to attack by pests is also an argument for a protective role (Van Damme *et al*., 1994). However, the direct evidence that lectins play a role in plant defense was obtained using purified protein in artificial diets or by using transgenic plants (Van Damme *et al*., 1994).

2.2.5.2 Anti-Insect Activity

Lectins have been suggested as one of the promising agents against insect pests and have been engineered successfully into a variety of crops including wheat, rice, tobacco, and potatoes (Velkov *et al.,* 2005). This approach could be used as a part of integrated pest management strategies and caveat pest attack. In general, it seems that large-scale implementation of transgenic insecticidal and herbicide-tolerant plants does not display considerable negative effects on the environment (Velkov *et al.,* 2005). Moreover, at least some transgenic plants can improve the corresponding environments and human health

because their production considerably reduces the load of chemical insecticides and herbicides (Velkov *et al.,* 2005).

Lectins demonstrate anti-insect activity by binding to chitin components in the insect gut, interacting with glycoconjugates on the surface of epithelial cells along the digestive tract, and binding to the sugar moiety of glycosylated digestive enzymes or assimilatory proteins and resistance to insect digestive proteases (Coelho *et al.,* 2007. They increase the mortality or delay the development of insect (*Kaur et al.,* 2006a). When incorporated in an artificial diet, *Arisaema jacquemontii* lectin adversely affected the development of *Bactrocera cucurbitae* larvae (*Kaur et al.,* 2006a). *Arisaema helleborifolium* lectin exhibited anti-insect activity towards the second instar larvae of *B. cucurbitae* (*Kaur et al.,* 2006b).

The insecticidal property of lectins may be due to orchestration of enzymatic activity of larvae (Chen *et al.,* 2009b). After treatment with different lectins, the activity of esterases in larvae was increased whereas the activity of acid phosphatase and alkaline phosphatase decreased (Chen *et al.,* 2009b). Galectin-1 treatment of *Plutella xylostella* larvae brought about disruption of the microvilli and induced abnormalities in epithelial cells (Chen *et al.,* 2009b). *Dioscorea batatas* lectin inhibited the emergence of *Helicoverpa armigera* larvae into adults by avidly binding to larval brush border and peritrophic membrane (Ohizumi *et al.,* 2009). *Arum maculatum* tuber lectin caused *Lipaphis erysimi* and *Aphis craccivora* to succumb, by binding to the gut brush border membrane vesicle proteins (Majumder *et al.,* 2005). *Olneya tesota* lectin bound to midgut glycoconjugates and microvillae of *Zabrotes subfasciatus* larvae (Lagarda-Diaz *et al.,* 2009). Diminished oviposition and a failure of emergence of adult beetles were observed (Lagarda-Diaz *et al.,* 2009). *Annona coriacea* lectin displayed toxicity in *Anagasta kuehniella* which apparently resulted from a change in the gut membrane environment and consequent

disruption of digestive enzyme recycling mechanisms by binding to midgut proteins (Coelho *et al.,* 2007). *Bauhinia monandra* leaf lectin produced mortality in *Zabrotes subfaciatus* and *Callosobruchus maculatus* when incorporated into an artificial diet (Macedo *et al.,* 2007). *Bauhinia monandra* leaf lectin bound to midgut proteins of the insect *Causus maculatus* (Macedo *et al.,* 2007). The detached leaves from transgenic tobacco plants expressing *Allium sativum* lectins reduced the weight gain and development and the metamorphosis of *Spodoptera littoralis* larvae (Sadeghi *et al.,* 2008). Furthermore, the larvae were detrimental to the pupal stage resulting in weight reduction and lethal abnormalities (Sadeghi *et al.,* 2008). Production of *Rhopalosiphum maidis* nymphs was significantly reduced on *Galanthus nivalis* agglutinin-expressing plants (Wang *et al.,* 2005). *G. nivalis* agglutinin was also found bound to glycoproteins that can be found in the guts of larvae of *Adalia bipunctata*, *Chrysoperla carnea*, and *Coccinella septempunctata* (Hogervorst *et al.,* 2006).

2.2.5.3 Antifungal Activity

Despite the large numbers of lectins and hemagglutinins that have been purified, only a few of them manifested antifungal activity (Sá *et al.,* 2009). The expression of *Gastrodia elata* lectins in the vascular cells of roots and stems was strongly induced by the fungus *Trichoderma viride*, indicating that lectin is an important defense protein in plants (Sá *et al.,* 2009). Following insertion of the precursor gene of stinging nettle isolectin I into tobacco, the germination of spores of *Botrytis cinerea*, *Colletotrichum lindemuthianum*, and *Trichoderma viride* was significantly reduced (Does *et al.,* 1999). Thus, lectins may be introduced into plants to protect them from fungal attack (Does *et al.,* 1999).

Plant lectins can neither bind to glycoconjugates on the fungal membranes nor penetrate the cytoplasm owing to the cell wall barrier (Parijs *et al.,* 1991). It is not likely lectins directly inhibit fungal growth by modifying fungal membrane structure and permeability

(Parijs *et al.,* 1991). However, there may be indirect effects produced by the binding of lectins to carbohydrates on the fungal cell wall surface. Chitinase-free chitin-binding stinging nettle (*Urtica dioica* lectin) impeded fungal growth (Parijs *et al.,* 1991). Cell wall synthesis was interrupted because of attenuated chitin synthesis andeposition (Parijs *et al.,* 1991). The effects of nettle lectin on fungal cell wall and hyphal morphology suggest that the nettle lectin regulates endomycorrhizal colonization of the rhizomes. Severa1 other plant lectins inhibit fungal growth. The first group includes small chitin-binding merolectins with one chitin-binding domain, e.g., hevein from rubber tree latex (Parijs *et al.,* 1991) and chitin-binding polypeptide from *Amaranthus caudatus* seeds (Broekaert *et al.,* 1992). The only plant lectins that can be considered as fungicidal proteins are the chimerolectins belonging to the class I chitinases. However, the antifungal activity of these proteins is ascribed to their catalytic domain.

2.2.5.4 Antibacterial Activity

The cell wall of bacteria not only precludes any interaction between the glycoconjugates on their membrane and carbohydrate-binding proteins but also prevents these proteins from penetrating the cytoplasm (Sequeira and Graham, 1977). Therefore, plant lectins cannot alter the structure and/or permeability of the membrane or disturb the normal intracellular processes of invading microbes. Therefore, if lectins play a role in the plant's defense against bacteria, it must be through an indirect mechanism that is based on interactions with cell wall carbohydrates or extracellular glycans. It has been suggested, for instance, that the potato lectin (which is considered as a cell wall protein) immobilized a virulent strain of *Pseudomonas solanacearum* in the cell wall (Sequeira and Graham, 1977). Virulent strains were not recognized by the lectin, escaped attachment to the cell wall, and therefore were able to multiply and spread over the plant (Broekaert and Peumans, 1986). Another indirect defense mechanism is the blocking of

the movements of normally motile bacteria at the air-water interface by the thorn apple (*Datura stramonium*) seed lectin (Broekaert and Peumans, 1986). By counteracting the chemotactic movement of soil bacteria toward the germinating seed, the lectin may prevent invasion of the seedling roots by potentially harmful bacteria. Since studies of the binding of plant lectins to bacterial cell wall peptidoglycans indicated that several legume seed lectins strongly interact with muramic acid, N-acetylmuramic acid, and muramyl dipeptide, the involvement of lectins in the plant's defense against microbes may have been underestimated (Ayouba *et al.*, 1994).

2.2.5.5 Antitumor Activity

It is well documented that lectins have an antitumor effect. *Flammulina velutipes* haemagglutinin-inhibited proliferation of leukemia L1210 cells (Ng *et al.* 2006). *Haliclona cratera* lectin displayed a cytotoxic effect on HeLa and FemX cells (Pajic *et al.,* 2002). Dark red kidney bean haemagglutinin exerted an antiproliferative activity toward leukemia L1210 cells (Xia and Ng, 2006). Small glossy black soybean (*Glycine max*) lectin impeded proliferation of breast cancer MCF7 cells and hepatoma HepG2 cells (Lin *et al.,* 2008). Del Monte banana lectin retarded proliferation of (L1210) cells and hepatoma (HepG2) cells (Cheung *et al.,* 2009). Extralong autumn purple bean lectin inhibited the proliferation of hepatoma HepG2 cells by inducing the production of apoptotic bodies (Fang *et al.,* 2010). Mistletoe lectin can be used in cancer patients to improve the quality of life (Semiglazov *et al.,* 2006).

In order to widen the application of anti-tumor lectins, the mechanism of action was elucidated. Lectins elicit apoptosis in different cancer cell lines. Examples include Korean mistletoe lectin-treated B16-BL6 melanoma cells (Park *et al.,* 2001), Korean mistletoe lectin-treated human A253 cancer cells (Choi *et al.,* 2004), *Agrocybe aegerita* lectin-treated HeLa cells (Zhao *et al.,* 2009), Abrus agglutinin-treated Dalton's

lymphoma cells (Bhutia *et al.*, 2008a) and HeLa cells (Bhutia *et al.,* 2008b), *Sophora flavescens* lectin-treated HeLa cells (Liu *et al.,* 2008), *Polygonatum odoratum* lectin-treated murine fibrosarcoma L929 cells (Liu *et al.,* 2009b), *Polygonatum cyrtonema* lectin-treated human melanoma A375 cells (Liu *et al.,* 2009a).

2.2.5.6 Antiviral Activity

The d-mannose-specific lectin from *Gerardia savaglia* was firstly reported to prevent infection of H9 cells with human immunodeficiency virus (HIV)-1 (Müller *et al.,* 1988). Furthermore, the lectin inhibited syncytium formation in the HTLV-IIIB/H9-Jurkat cell system and HIV-1/human lymphocyte system by reacting with the oligosaccharide side chains of the HIV-1 gp120 envelop molecule (Müller *et al.,* 1988). The lectins concanavalin A, wheat germ agglutinin, *Lens culinaris* agglutinin, *Vicia faba* agglutinin, *Pisum sativum* agglutinin and phytohaem (erythro) agglutinin were found to bind to gp120 (Hansen *et al.,* 1989). They inhibited fusion of HIV-infected cells with CD4 cells by a carbohydrate-specific interaction with the HIV-infected cells (Hansen *et al.,* 1989). Plant lectins displayed anti-coronaviral activity, especially mannose-binding lectins, in severe acute respiratory syndrome coronavirus (Keyaerts *et al.,* 2007). They interfered with viral attachment in early stage of replication cycle and suppressed the growth by interacting at the end of the infectious virus cycle (Keyaerts *et al.,* 2007). Banana (*Musa acuminata*) lectin inhibited HIV replication (Swanson *et al.,* 2010).

The treatment of AIDS with lectins was further investigated. Different lectins have different anti-HIV mechanisms. Lectin from the polychaete marine worm *Chaetopterus variopedatus* inhibited cytopathic effect induced by HIV-1 and the production of viral p24 antigen (Wang *et al.,* 2005). The sea worm (*Serpula vermicularis*) lectin suppressed the production of viral p24 antigen and cytopathic effect induced by HIV-1 (Molchanova *et al.,* 2007). *Polygonatum cyrtonema Hua* lectin inhibited HIV-I- and HIV-II-induced

cytopathicity in MT-4 and CEM cells (An *et al.,* 2006). Banana lectin directly bound the HIV-1 envelope protein (gp120) and blocked entry of the virus into the cell, and decreased the levels of the strong-stop product of early reverse transcription (Swanson *et al.,* 2010). Extra long autumn purple bean lectin (Fang *et al.,* 2010) and mushroom *Russula delica* lectin (Zhao *et al.,* 2009) were able to inhibit HIV-1 reverse transcriptase. Hence, lectins are potential drugs for treatment of AIDS.

2.3 *Culex quinquefasciatus*

Culex quinquefasciatus is a vector of *Wuchereria bancrofti* in the urban areas of several countries in the world along the coastal plain. Lymphatic filariasis is transmitted in both urban and rural areas by *Culex quinquefasciatus* (WHO, 1998). *Culex quinquefasciatus* are the most prevalent biting mosquitoes occurring in high densities in nearly all residential areas of cities. Resistance to chlorinated hydrocarbons, organo-phosphorus compounds and carbamates has been reported from a number of countries (WHO, 1988). In addition, *Culex quinquefasciatus* constitutes a considerable nuisance in all tropical and subtropical urban areas, disrupting the inhabitants' sleep to the extent of causing fatigue from lack of rest (WHO, 1988). Nearly all breeding sites of *Culex quinquefasciatus* in urban areas are man-made structures such as blocked drains, septic tanks, cesspools, pit latrines and other sources of stagnant polluted water such as small discarded containers (WHO, 1988). *Culex quinquefasciatus* has not yet been incriminated as a vector in central and West Africa. Its proliferation, as a result of urbanization, increases its importance as a pest (WHO, 1988).

2.3.1 Taxonomic Hierarchy

Kingdom	-	*Animalia*
Phylum	-	*Arthropoda*
Subphylum	-	*Hexapoda*
Class	-	*Insecta*
Subclass	-	*Pterygota*
Infraclass	-	*Neoptera*
Order	-	*Diptera*
Suborder	-	*Nematocera*
Infraorder	-	*Culicomorpha*
Family	-	*Culicidae*
Subfamily	-	*Culicinae*
Tribe	-	*Culicini*
Genus	-	*Culex*
Species	-	*Culex quinquefasciatus* (*say*) (Anoymous, 2009).

2.3.2 Means of Dispersal of Mosquitoes

Worldwide introduction of various mosquito species over large distances into regions where they are not indigenous has occurred through human agencies, primarily on sea routes, in which the eggs, larvae, and pupae inhabiting water-filled used tires and cut flowers are transported. However, apart from sea transport, mosquitoes have been effectively carried by personal vehicles, delivery trucks, and trains and aircraft. Quarantine measures have proved difficult to apply sufficiently consistently (www.debate.org 2012).

2.3.3 The Feeding Habits of Mosquitoes

The male and female mosquitoes take sugar meals from plants (Clements, 1992). Following mating, the female seeks a blood meal (Clements, 1992). *Culex quinquefasciatus* are opportunistic feeders, feeding on mammals and birds throughout the night (Clements, 1992). The males survive only on sugar meals, while the female will take multiple blood meals. Most female mosquitoes have to feed on an animal and get a sufficient blood meal before she can develop eggs; this process is known as anautogenous reproduction. If they do not get this blood meal, then they will die without laying viable eggs.

2.3.4 Flight Habits of Mosquitoes

The flight range for females is usually longer than that of males *www.mosquitoes.org/LifeCycle.html* 2007. Many times wind is a factor in the dispersal or migration of mosquitoes *www.mosquitoes.org/LifeCycle.html* 2007. Most mosquitoes stay within a mile or two of their source. However, some have been recorded as far as 75 miles from their breeding source (www.mosquitoes.org/LifeCycle.html 2007).

2.3.5 Life Cycle of *Culex quinquefasciatus*

Gravid *Culex quinquefasciatus* females fly during the night to nutrient-rich standing water where they lay their eggs. They oviposit in waters ranging from waste water areas to bird baths, old tires, or any container that holds water. If the water evaporates before the eggs hatch or the larvae complete their life cycle, they die (Mosquito Information website, 2007).

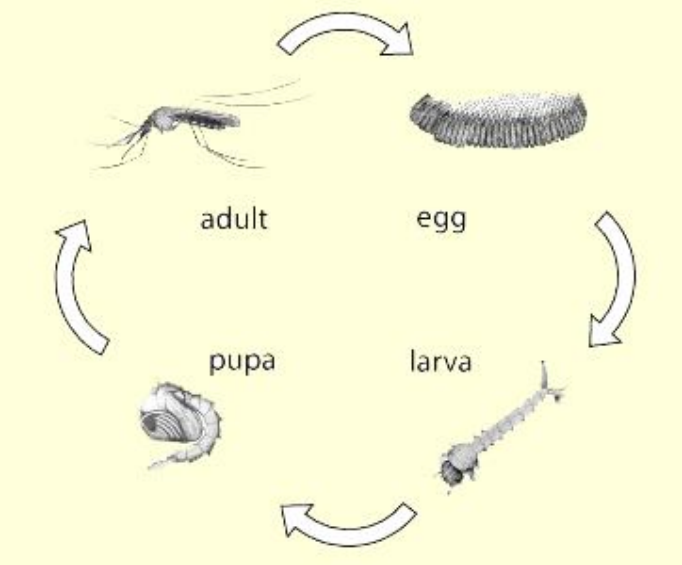


Figure 2.2: The Life Cycle of a mosquito (McCafferty, 1983)

2.3.6 The Egg

The eggs of *Culex quinquefasciatus* are laid in rafts loosely cemented together with 100 or more eggs in a raft. A raft of eggs looks like a speck of soot floating on the water and is about ¼ inch 2long and 1/8 inch wide which will normally hatch 24 to 30 hours after being oviposited (Bates, 1949). The eggs are laid on the surface of fresh or stagnant water which may be in tin cans, barrels, horse troughs, ornamental ponds, swimming pools, puddles, creeks, ditches, or marshy areas. *Culex quinquefasciatus* usually lay their eggs at night (Spielman *et al*., 2001). A mosquito may lay a raft of eggs every third night during its life span (Spielman *et al*., 2001).



Figure 2.3: The *Culex quinquefasciatus* Egg Raft (McCafferty, 1983)

2.3.7 The Larva

Mosquito larvae, commonly called "wrigglers" because they swim either through propulsion with their mouth brushes, or by jerky movements of their entire bodies, must live in water from 7 to 14 days depending on water temperature. Larvae must come to the surface at frequent intervals to obtain oxygen through a breathing tube called a siphon; they dive below the surface only when disturbed. The larval head is short and stout becoming darker toward the base. The mouth brushes have long yellow filaments that are used for filtering organic materials. The abdomen consists of eight segments, the siphon, and the saddle. Each segment has a unique setae pattern (Sirivanakarn and White, 1978). The siphon which is on the dorsal side of the abdomen is four times longer than it is in other species with multiple setae tufts (Darsie and Morris, 2000). The saddle is barrel shaped and located on the ventral side of the abdomen with four long anal papillae protruding from the posterior end (Sirivanakarn and White, 1978). Larvae develop through four stages, or instars, after which they metamorphose into pupae. At the end of each instar, the larvae molt, shedding their skins to allow for further growth (Spielman *et al.*, 2001).

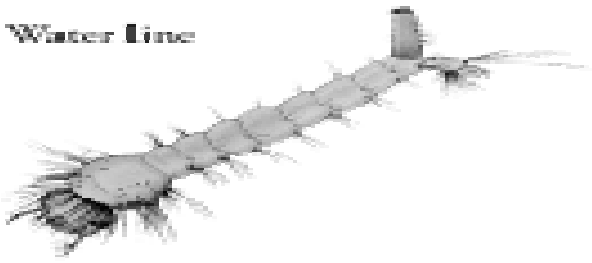


Figure 2.4: *Culex quinquefasciatus* Larva breathing in atmospheric oxygen from the water surface (McCafferty, 1983)

2.3.8 The Pupa

Mosquito pupae are comma-shaped, and are commonly called "tumblers". The head and thorax are merged into a cephalothorax, with the abdomen curving around underneath. The pupa is less active than the larva; they must live in water from 1 to 4 days, depending upon species and temperature (Spielman *et al*., 2001). The pupa is lighter than water and therefore floats at the surface (Spielman *et al*., 2001). It takes oxygen through two breathing tubes called "trumpets" on the cephalothorax. When it is disturbed it dives in a jerking, tumbling motion and then floats back to the surface (Spielman *et al*., 2001). However, pupae do not feed during this stage (Spielman *et al*., 2001). The metamorphosis of the mosquito into an adult is completed within the pupal case, after a few days, the pupa rises to the water surface, the dorsal surface of the cephalothorax of the adult mosquito splits the pupal case and emerges to the surface of the water where it rests until its body can dry and harden (Spielman *et al*., 2001).

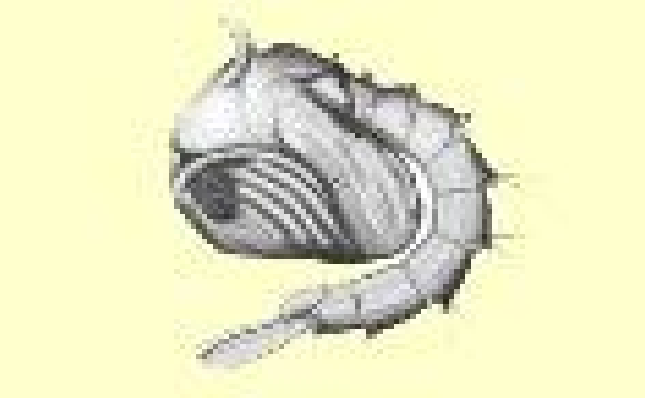


Figure 2.5: *Culex quinquefasciatus* Pupa (McCafferty, 1983)

2.3.9 The Adult

The adult *Culex quinquefasciatus* vary from 3.96 to 4.25 mm in length (Lima, *et al*., 2003). The mosquito is brown with the proboscis, thorax, wings, and tarsi darker than the rest of the body. The head is light brown with the lightest portion in the center. The antennae and the proboscis are about the same length, but in some cases the antennae are slightly shorter than the proboscis. The flagellum has thirteen segments that have few to no scales (Sirivanakarn *et al.,* 1987). The scales of the thorax are narrow and curved. The abdomen has pale, narrow, rounded bands on the basal side of each tergite. The bands barely touch the basolateral spots taking on a half-moon shape (Darsie and Ward 2005).



Figure 2.6: An Adult *Culex quiquifasciatus* (McCafferty, 1983)

2.4 LARVICIDES

Insecticides are agents of chemical or biological origin that control insects; they include ovicides and larvicides used against the eggs and larvae of insects respectively. Insecticides are used in agriculture, medicine, industry and the household. A larvicide is an insecticide that is specifically designed to target the larval stage of an insect. Their most common use is against mosquitoes (van Emden and Pealall, 1996). Larvicides may be contact poisons, stomach poisons, growth regulators, or (increasingly) biological control agents (van Emden and Pealall, 1996). The use of insecticides is believed to be

one of the major factors behind the increase in agricultural productivity in the 20th century (van Emden and Pealall, 1996). Nearly all insecticides have the potential to significantly alter ecosystems; many are toxic to humans; and others are concentrated in the food chain (van Emden and Pealall, 1996). Control may result from killing the insect or otherwise preventing it from engaging in behaviors deemed destructive (George and David, 2004). Insecticides may be natural or man-made and are applied to target pests in a myriad of formulations and delivery systems (sprays, baits, slow-release diffusion and so on). The science of biotechnology has, in recent years, even incorporated bacterial genes coding for insecticidal proteins into various crop plants that causes the death of unsuspecting pests that feed on them (George and David, 2004).

Humanoids have been on earth for more than 3 million years, while insects have existed for at least 250 million years (Carpenter and Ware, 2004). We can guess that among the first approaches used by our primitive ancestors to reduce insect annoyance was hugging smoky fires or spreading mud and dust over their skin to repel biting and tickling insects, a practice resembling the habits of elephants, swine, and water buffalo. Today, such approaches would be classed as repellants, a category of insecticides (Carpenter and Ware, 2004). Historians have traced the use of pesticides to the time of Homer around 1000 B.C., but the earliest records of insecticides pertain to the burning of "brimstone" (sulfur) as a fumigant. Pliny the Elder (A.D. 23-79) recorded most of the earlier insecticide uses in his *Natural History*. Among these were the use of gall from a green lizard to protect apples from worms and rot. Later, we find a variety of materials used with questionable results: extracts of pepper and tobacco, soapy water, whitewash, vinegar, turpentine, fish oil, brine, lye among many others. At the beginning of World War II (1940), insecticide selection was limited to several arsenicals, petroleum oils, nicotine, pyrethrum, rotenone, sulfur, hydrogen cyanide gas, and cryolite (Carpenter and

Ware, 2004). It was World War II that opened the *Modern Era of Chemical* control with the introduction of a new concept of insect control synthetic organic insecticides, the first of which was DDT (Thomson, 2000).

2.4.1 Insecticides of Plant Origin

Botanicals are a promising source of pest control compounds. The pool of plants possessing insecticidal substances is enormous (Jacobson, 1954). These have generated extraordinary interest in recent years as potential sources of natural insect control agents. Over 2000 species of plants are known that possess some insecticidal activity (Jacobson, 1989). The first insecticides to be used by man were from plants, the biological activities of which were known from the earliest recorded times (Smith, 1975). In the middle of the 17th century, pyrethrum, nicotine, and rotenone were recognized as effective insect-control agents (Crosby, 1966). Many plant species have been known to contain relatively high amount of secondary metabolites with probable insecticidal, repellant, anti-feedant or insect growth regulating activity. These include *Adansonia digitata, Cyperus rotundus, Parkia clappertonia and Annona senegalensis* (Abubakar and Abdurahaman, 1998). Among others is *Stegonotaenia araliacea* which is well used in Northern Nigeria for insect pest control. This plant has a wide range of activity against A*edes aegypti* and *Culex quinquefasciatus* and may be a good potential source of plant derived anti-insect products (Garba *et al.,* 1997). Triterpenes have a wide range of anti-feedant activity against insects as shown by the triterpenes of *Azadirachta indica* (Akou- Edi, 1984). The rhizomes of *Cyperus articulatus* when incinerated around dwelling places drive away insects (Dalziel, 1995). *Cyperus articulatus* also contains constituents that causes nausea in insects especially terpenes that are often responsible for insect repellent and antifeedant actions. The terpenoids present in this plant are mainly mono and sesqul (major constituents of essential oils) in nature, with aromatic smell. While their harmful

effect to man and other mammals is unheard, they repel insects (Abubakar, 1995). *Cyperus rotundus* was reported to contain chemical compounds having insecticidal actions (Abubakar, 1995). Hellebore is another plant with insecticidal property. Insecticide is usually prepared from the rhizomes of white hellebore (*Veratrum album*). It has also been shown that the rhizomes of green hellebore (*Veratrum vivide*) are equally effective (Idem, 1924). *Veratrum album* and *Veratrum vivide* have been found to contain several alkaloids with quite appreciable insecticidal properties (Idem, 1924). *Veratrum album* has been reported to act as contact insecticide, mainly used against biting insects on fruits (Imperial Institute, 1940).

2.5 MECHANISM OF INSECT RESISTANCE TO CHEMICAL INSECTICIDE

The susceptibility of insect vectors to insecticides has been widely studied in different locations of the world. Wide varieties of insects have been used as test models. As at 1992, the list of insecticide-resistant vector species included: body lice, 9 species of ticks, sand flies, 8 species of fleas, bedbugs, triatomoids, 56 species of Anopheles and 39 species of culicines (Brodgon and Mc Allister, 1998). Resistance has spread to every chemical class of insecticide including microbial drugs and insect growth regulators (Brodgon and Mc Allister, 1998). Insects have evolved three major mechanisms to overcome toxicants as discussed below.

2.5.1 Biochemical Resistance

In this form of resistance, an insecticide is detoxified by one or more enzymes before it can reach its site of action (Brodgon, and MC Allister, 1998). Mixed-function oxidases or other enzymes are involved (Brodgon, and MC Allister, 1998). The two major forms of biochemical resistance are target- site resistance and detoxification enzyme- based resistance (Brodgon, and MC Allister, 1998).

2.5.1.1 Target Site Resistance

Target site resistance occurs when the insecticide no longer binds to its target as a result of alteration of amino acid responsible for insecticide binding at its site of action (Brodgon, and MC Allister, 1998). The target of organophosporus and carbamate insecticide is acetyl cholinesterase in nerve synapse and the target of organochlorine and synthetic pyrethroids is the sodium channel of the nerve sheath (Brodgon, and MC Allister, 1998). According to Miyazaki *et al.* (1996), DDT- Pyrethroid cross-resistance may be produced by single amino acid changes in the axonal sodium channel insecticide binding site.

2.5.1.2 Detoxification Enyme Based Resistance

Detoxification enyme based resistance occurs when enhanced levels or modified activities of esterases, oxidases, glutathione s-transferases (GST) prevent the insecticide from reaching its site of action Brodgon and Mc Allister, 1998). For instance Cytochrome P450 monooxygenases is associated with pyrethroid resistance in German cockroaches (Pridgeon *et al*., 2002), *Culex quinquefasciatus* (Chandre *et al.,* 1998), *Anopheles funestus* and *Anopheles gambiae* s.s (Etang *et al.,* Nikou *et al.,* 2003). However, the most common resistance mechanisms in insects are modified levels of activity of esterases, detoxification enzymes that metabolise a wide range of insecticides (Brodgon and Mc Allister, 1998).

2.5.2 Physiological Resistance

Physiological resistance is any form of resistance that reduces toxicity through changes in basic physiology (Brodgon and Mc Allister, 1998). In this form of resistance, the chemical is not broken down into a less toxic form; rather the insect accommodates the chemical by altering one or more physiological functions (Brodgon and Mc Allister, 1998). This may involve reduced neuronal sensitivity to insecticides e.g., knockdown

resistance (kdr) in house flies to Dichloro Diphenyl Trichloroethane and pyrethroids; altered acetylcholinesterase. Acetylcholinesterase that is less sensitive to inhibition by organophosphorus and carbamate insecticides has been documented in resistant strains of several insect, tick and mite species. e.g. *Anopheles* mosquitoes resistant to organophosphorus and carbamates have been shown to have an altered acetylcholinesterase; decreased penetration of the insecticide through the body wall through modification of the structure or composition of the cuticle (Brodgon and Mc Allister, 1998). For example, additional waxy layers in resistant strains; and increased excretion or sequestration of insecticide as seen in Dichloro Diphenyl Trichloroethane storage in body fat, preventing it from reaching the site of action (Brodgon, and MC Allister, 1998).

2.5.3 Behavioural Resistance

Involves changes in behaviour by which mosquitoes avoid insecticides e.g *Anopheles gambiae*, an endophillic strain (indoor dwelling) was susceptible to DDT sprays applied to the indoor walls. An exophillic strain, not inhabiting indoors, became dominant because its behavior allowed it to avoid exposure to the insecticide (Brodgon, and MC Allister, 1998).

2.6 MOSQUITO CONTROL

Mosquito control can be directed at either the immature aquatic stages or the adults or at both stages simultaneously.

2.6.1 Biological Control Method Directed at the Aquatic Stage

2.6.1.1 Use of Predators

Mosquito larvae and pupae have been reduced in aquatic environments by the use of predators (Collins and Blackwell, 2000). The commonly used predators are fish, example

Gambusia species. Other predators are tadpoles of frogs and toads, and various aquatic insect larvae, but these have rarely proved effective control agents (Collins and Blackwell, 2000). A few mosquitoes have predacious larvae, for example *Toxohynchites brevipalpis* Theo that are used to control larvae of other container- breeding mosquitoes (Collins and Blackwell, 2000).

2.6.1.2 Chemical Control Directed at Adult Mosquito

A principal strategy for controlling malaria is to use insecticides to eradicate the vectors (WHO, 1993). The most widely practiced method of chemical control targeted at the adult mosquito is the spraying of insecticides to the interior surface of walls, ceilings, and roofs of houses to control the endophagic mosquito species and also spraying insecticides outside houses, windows, open shades and the surrounding of human or animal habitation to control the exophagic mosquito species (WHO, 1993). The use of insecticide- treated mosquito nets and other materials are being used currently as vector control measures against mosquitoes. Pyrethroids (Deltamethrin permethrin) and Dichloro, diphenyl, trichloroethane (DDT) are used to treat bed nets and insect window nets (WHO, 1993). Before the advent of resistance to insecticides by mosquito, DDT was the best insecticide. Organochlorine insecticides like Dieldrin (cyclodiene) and HCH, which are more toxic to man than DDT, are also for indoor residual spraying under strict safety measures (Service, 1980). If mosquitoes are resistant to DDT then organophosphate insecticides such as malathion, fenthion (Baytex), Fenitrothion (sumithion) or carbamates, such as carbaryl (Sevin) and Propoxur (Arprocarb) are used (Service, 1980). But they are less persistent and spraying may have to be repeated severally (4-6 times) in a year. Furthermore, they are more expensive than DDT and are toxic to man, thus greater safety measures may have to be taken during control campaigns, (Service, 1980). In some countries, where local malaria vector is still

susceptible, DDT is used for indoor residual spraying. However, in almost all countries, DDT has been banned due to its adverse toxic effects on man, animal, agriculture, earth's resources and biodiversity (WHO, 2000).

2.6.1.3 Environmental Management Control

Environmental management for vector aims to induce changes in the ecosystem that help reduce their receptivity to the propagation of disease vectors. It is not intended to replace other control strategies; rather, it provides a basis for other methods, such as chemical control to build on in a complimentary fashion, while reducing the environmental cost and resistance risks incurred by excessive use of insecticides (WHO, 1982). Unfortunately environmental management fell off the Malaria Control Agenda when DDT became the main tool for the World Health Organisation's (WHO) Malaria eradication programme from 1957-1967 (Service, 1980). Thus the effectiveness of environmental management has remained tragically under exploited, (WHO, 1993). Environmental management for malaria vector controls consists of options for reducing the number of mosquito habitats or reducing biting by adult mosquitoes (Service, 1980). A simple form of control consists of filling in of all available mosquito larval habitats with soil and gracels. Examples of larval habitat range from water-filled tree holes, ponds to small marshes. Abandoned cans, metal drums, pots and tyres also other containers or receptacle of water can constitute a good habitat for mosquitoes breeding. The water in such containers should be thrown away; Larval breeding places such as large ponds burrow pits fresh or salt water marshes can be drained. The advantage is that, it can lead to permanent control of larvae or pupae. Ecological changes can also prevent mosquito breeding; for example, cutting down over hanging vegetation to increase sunlight on the water may prevent breeding by species that like shaded habitats; whereas planting vegetation near water may stop breeding by sun–loving species. Another method of

controlling mosquito larvae is by the application of mineral oils to the water. Larval habitats have to be treated with chemicals such as Paris green oils and organophosphate chemicals are toxic to other aquatic life and are consequently not so widely used, due to its health implication to aquatic life and to man (Service, 1980).

CHAPTER THREE

MATERIALS AND METHOD

3.1 MATERIALS

3.1.1 Plant Material

The plant stem bark and leaves were collected from Kagarko in Kaduna State. The plant was identified to be E*rythrophleum suaveolens* with voucher number 242 at the Herbarium, Botany Unit, Ahmadu Bello University, Zaria, Kaduna state.

3.1.2 Blood Sample Collection

The blood samples used for haemagglutination assay were collected from a blend of chickens slaughtered in Samaru chicken market in 5mls EDTA sample bottles.

3.1.3 Larval Collection and Authentication

The mosquito larvae were harvested from their natural breeding sites which were stagnant water in gutters and water ponds within the campus of Ahmadu Bello University, Zaria. The harvested larvae were transported to the Entomology Laboratory of the Biological Science Department, Ahmadu Bello University (A.B.U), Zaria, and identified to be *Culex quinquefasciatus*.

3.1.4 Equipment and Analytical Instruments

Electrophoresis unit EPS 601 (Amersham, Pharmacia Biotech), UV/visible spectrophotometer (JENWAY 6305), Centrifuge (JOUAN GR2022 and Labofuge 300 Heraeus), Chromatography column (9x2cm^3), Weighing balance (AND GF-2000), pH meter (JENWAY 3150), Water bath (GRANT JB SERIES), Oven (HARVARD / LTE VALCAN), Micropipettes (100-100µl Erma Optical Works Ltd. Tokyo, Japan), Micro titer plates, Thermometer, Mosquito Cages, Plastic bowls (500mls), Mosquito nettings, Plastic aspirator, Cotton wool, Rubber band, quartz cuvette.

3.1.5 Chemicals and Reagents

All reagents used were of analytical grade obtained from Sigma Chemical Company, England. They included, Ammonium Sulphate, Sephadex G-75, Silver nitrite staining kit-Silver Nitrite^{TM}, Sp- sephadex C-50, Protein markers, Sodium dodecyl sulphate, Sucrose, Glucosamine, Glucose, Maltose, Lactose, Galactose , Arabinose, Raffinose, Maltose ,Mannose, Sulphuric acid, phenol, Commasie brilliant blue R-250,TEMED, Bromophenol blue Phosphoric acid, glycerol, Tris, Hydrochloric acid, acrylamide, bisacrylamide, Glycine, Ammonium per sulphate (APS).

3.2 METHODS

3.2.1 Extraction

The *Erythrophleum suaveolens* bark was dried to a constant weight in an oven at a temperature of (45°C) for four days. The dried bark was pulverized into powdered form with a wooden mortar and pestle. A 25g portion of *Erythrophleum suaveolens* powder was extracted in 500mls of 0.02M phosphate buffer pH 7.2 at 4°C. (1:20 w/v) at room temperature for 24 hours. The suspension was filtered through muslin cloth and then the filtrate was centrifuged at 8000g for 15 min at 4°C (Shangary *et al.,* 1995). Then 500ml of the clear supernatant was collected as crude saline extract and assayed for haemagglutination activity and total protein content.

3.2.2 Washing of Erythrocyte Cells

The collected blood samples were centrifuged at 3000g on a Bench centrifuge for 10 minutes at room temperature. The supernatants (plasma and leucocytes) were carefully removed with the aid of a micro pipette, while the packed red blood cells were washed in 10ml 0.2M phosphate buffered saline (PBS) pH 7.2 in a 50ml centrifuge tube. The process of washing and centrifugation were repeated five times until the supernatants

were clear. Then, 2% v/v chicken erythrocytes were prepared in 0.2M phosphate buffered saline (PBS) pH 7.2 as described by Oyedapo *et al.,* (2004).

3.2.3 Haemagglutination Assay

The haemagglutination assay (HA) was carried out as described by Calderón de la Barca *et al*. (1985). Exactly 100 µl of phosphate buffered saline (PBS) pH 7.2 solutions were added to each well of a 96-well micro-titer plate, and then a 100 µl of the *Erythrophleum suaveolens extract* was transferred to the first well. Serial dilutions were prepared by transferring a 100 µl aliquot of a more concentrated sample to adjacent wells containing diluents alone. It was mixed and dilution procedure continued. An aliquot (100 µl) of 2% (v/v) suspension of the prepared erythrocytes was added to each well and the micro-titer plate was maintained at room temperature. The haemagglutination was observed visually after one hour, and the results were expressed in haemagglutination units (HU). Haemagglutination unit (HU) was defined as the reciprocal of the maximum dilution presenting visible agglutination of red blood cells (that is, if the 1/4 titre represented the maximum dilution exhibiting agglutination and the volume of sample assayed was 100 µl, then the HA value would be 4 HU/100 µl). Negative control samples consisted of phosphate buffered saline solution and erythrocyte suspension only.

3.2.4 Protein Determination

A Standard curve was made using a stock solution of bovine serum albumin (BSA) to prepare a protein solution of concentrations of 1-10 mg / ml. The protein content was determined as described by Bradford (1976). The Bradford reagent was diluted five-fold with distilled water (1 part Bradford: 4 parts water) and filtered through whatman filter paper. Then 1ml portions were dispensed into 14 test tubes followed by 20 µl of the *Erythrophleum suaveolens* lectin, it was allowed to stand for 30 minutes before readings were taken at 595nm wavelength.

3.2.5 Partial Purification of E*rythrophleum suaveolens* Lectin

3.2.5.1 Ammonium Sulphate Precipitation

Enough solid ammonium sulphate (57g) was slowly added with stirring to the crude extract (500ml) of E*rythrophleum suaveolens* to make it 20% saturated. The mixture was centrifuged at 3000rpm for 15minutes at 4°Cand the supernatant (600ml) was carefully collected. To the supernatant solid ammonium sulphate (312g) was added to 20% saturated supernatant (600ml) to make it 90% saturated. The mixture was centrifuged as above and the supernatant was discarded. The precipitate was dissolved in 2ml of phosphate buffered saline (PBS) pH 7.2 (Cooper, 1942; Pereira *et al*., 2008).

3.2.5.2 Gel Filtration Chromatography

Gel filtration was carried out as described by Whittaker (1963) and Andrews (1964). A 9 x $2cm^3$ column was packed with swollen sephadex G-75 resin and equilibrated with 0.02M Phosphate saline buffer pH 7.2 to remove traces of impurity and desalt NH_4SO_4 contained in the lectin pellet. The lectin pellet was re-suspended in 5ml of 0.02M Phosphate saline buffer pH 7.2. Subsequently, 1ml of the lectin solution was loaded onto the column and eluted with phosphate saline buffer at a flow rate of 7ml / minute and 40 fractions were collected (5ml of eluent each). After reading the absorbance at 280nm the eluent fractions (of 5ml) were assayed for lectin activity by haemagglutination assay.

3.2.5.3 Ion Exchange Chromatography

Ion exchange chromatography was carried out as described by Knight, (1967) using Sp-Sephadex C-50 cation exchanger. The column was pre-equilibrated with 0.02M Phosphate buffer pH 7.2. Then, 2ml of the sample from gel filtration was loaded and the column was eluted with NaCl gradient (0.01- 0.3M) at a flow rate of 4ml / 5mins. A total of 25 fractions of 5mls each were collected and assayed for haemagglutination activity and total protein.

3.2.5.4 Sodium Dodecyl Sulphate Polyacrylamide-Gel Electrophoresis (SDS – PAGE)

Electrophoresis was carried out on E*rythrophleum suaveolens* crude lectin and active eluates from sephadex G-75 and Sp-Sephadex C-50 as described by Laemmil (1973) using a 4.5% (w/v) acrylamide stacking gel and a 12.5% (w/v) acrylamide separating gel. The gel was calibrated with broad range molecular markers β-Galactose (120kDa), Bovine serum albumin (94kDa), Ovalbumin (47kDa) Carbonic Anhydrase (37kDa), Myoglobin (28kDa), and Lysozyme (19kDa). Exactly, 50µl of Ammonium per sulphate (APS) and 2µl of TEMED were added to 2ml of 30% acrylamide solution to initiate gel polymerization after which the solution was poured into the electrophoresis apparatus already containing the running buffer (3.03g Tris, 14.04g Glycine and 1g SDS in water). About 100µL of crude and purified *Erythrophleum suaveolens* lectin were pre-treated with one drop 2% (w/v) 2-mercaptoethanol, one drop of bromophenol blue and 50µL of Tris – HCL buffer pH 6.8, it was then heated for 5mins at 95°C after which three drops of glycerol were added to the crude and purified *Erythrophleum suaveolens* lectins which were to be subjected to electrophoresis. Exactly 5 µl crude and partially purified lectin and the standard proteins were loaded separately on the gels and electrophoresis was ran at a current of 20mA with Tris- glycine buffer pH 6.8 was used as the running buffer. The gel was silver stained by the method of Vari and Bell (1996). This involved six steps which includes the use of gel fixing solution1 to remove all interfering ions by placing the gel in a staining tray and adding 100ml of the solution to the gel and micro-waving for 30 seconds, after which the staining tray was agitated for 1 hour and the gel fixing solution1 was discarded. The gel fixing solution 2 was used to remove acetic acid from step 1, this was done by adding 100ml of fixing solution 2 to the gel and micro waving for 30 seconds and agitating the tray for 20 minutes, the gel was washed twice with 100ml of de-ionzed water and agitated for 20 seconds, sensitizing and washing of the gel

was done to increase the sensitivity and contrast of staining by adding 100ml of sensitizing solution and agitating for 1 minute. The gel was washed twice with 100ml of de-ionized water and agitated for 20 seconds. Staining was done by adding 100ml of the staining solution and agitating for 20 minutes and washing with 100ml of de-ionized water for 20 seconds, the gel was developed by adding 100ml of the developing solution and agitating for 10 minutes until bands developed and termination of the fixing process was done by adding 100ml of the stop solution and agitating for 10 minutes and gel was de-stained for visualization.

3.2.6 Partial Characterisation of Crude and Purified *Erythrophleum suaveolens* Lectins

3.2.6.1 Determination of Sugar Specificities of Crude and Purified *Erythrophleum suaveolens* Lectins

The inhibitory effects of various sugars on the haemagglutinating activity of chicken erythrocyte by the isolated lectins were determined as described by Kuku and Eretan (2004).

For each sugar, 50µL each of 1M and 0.5M were placed in separate microtitre plates wells. This was followed by the addition of 50µL of the crude and purified lectins to each well. Then 50µL of 2% (v/v) erythrocyte suspension was added to each mixture, mixed and monitored for agglutination for 1 hour. The sugars used for this experiment were: glucosamine**,** glucose, maltose, lactose, galactose, arabinose, raffinose, maltose , mannose and xylose.

3.2.6.3 Thermostability of Crude and Purified *Erythrophleum suaveolens* Lectin

The effect of temperature on the haemagglutination activity was monitored as described by Sampaio *et al.,* (1998). Aliquots of *Erythrophleum suaveolens* lectin were incubated in water bath for 30 minutes at different temperatures (30, 40, 50, 60, 70, 80, 90°C).

The heated solution was rapidly cooled on ice and assayed for haemagglutination activity. The haemagglutination activity of the control that was kept at 20°C for 30 minutes was used as reference.

3.2.6.3 Effect of pH on Haemagglutination Activity of Crude and Purified *Erythrophleum suaveolens* Lectin

The effect of pH on the haemagglutinating activity was determined by carrying out the haemagglutinating assay of the lectins using the following buffers at different pH values; 0.2M glycine-NaOH buffer, pH 10-12; 0.2M citrate-phosphate buffer, pH 3-7, and 0.2M borate buffer, pH 7.5-9.5. The control values were the hemagglutination titre of the lectin using 0.2M Phosphate buffereda saline, pH 7.2.

3.2.6.4 Determination of Total Carbohydrate of Crude and Purified *Erythrophleum suaveolens* Lectin

The total sugar concentration of crude and purified *Erythrophleum suaveolens* lectin was determined by phenol sulphuric acid method as described by Dubios *et al*., (1956). A sugar standard was prepared using 1 mg/ml fructose standard solution, in distilled water. Aliquots of fructose standard solution were transferred to 10 different test tubes in 5-increments ranging from 5 to 50 µl with a pipette. Then 10µl of the sample to be analyzed was transferred into a test tube, exactly 500 µl of 4% phenol and 2.5 ml 96% sulfuric acid were added to all the tubes to break all the glycosidic linkages and the coloured complex was formed in this step. The solutions were transferred from the test tubes to the cuvettes and absorbance read at 490nm. The concentration of sugar present in the sample was estimated by plotting a graph of 490nm versus sugar concentration of the sugar calibration standard.

3.2.7 Quantitative phytochemical composition of crude and purified *Erythrophleum suaveolens* lectin

3.2.7.1 Determination of Alkaloid

The gravimetric method of Harbone (1980) was adopted in the determination of alkaloid content. Exactly 5mls each of the extract were weighed in to a 250ml beaker and 200ml of 10% acetic acid in ethanol were added and allowed to stand for 4 hours. Extract was then filtered and concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop-wise to the extract until precipitation was completed. The whole solution was allowed to settle, and the precipitate filtered in an already weighed filter paper washed with 1% NH_4OH solution and was dried in an oven.

3.2.7.2 Determination of Saponin

A gravimetric method of AOAC (1984) employing the use of soxhlet extractor and two different organic solvent was used. The first solvent extracted lipids and interfering pigments, while the second solvent extracted Saponin. 2mls of each of the extracts were weighed in to a thimble and put in a soxhlet extractor with a condenser fitted on top. Extraction was done with acetone in a 250ml round bottom flask for 3hrs, after which another weighed 250ml round bottom flask containing methanol was fitted to the same extractor and extraction continued for another three hours. At the end of the second extraction, the methanol was recovered by distillation and the flask oven-dried to remove the remaining solvent in the flask. The flask was allowed to cool in a desiccator and its weight taken.

3.2.7.3 Estimation of total phenolic content

The total phenolic content in crude and purified *Erythrophleum suaveolens* lectins was estimated by Folin-Ciocalteau reagent as described by Singleton and Rossi (1965). Gallic acid stock solution (1000µg/ml) was prepared by dissolving 100mg of gallic acid in

100ml of ethanol. Various dilutions of standard gallic acid were prepared from this stock solution. Folin-Ciocalteau reagent was prepared by mixing Folin's reagent with Phenol reagent (1:1), and diluted 1:1 in distilled water, before use. A Calibration curve was plotted by mixing 1ml aliquots of 1.0, 2.5, 5.0, 10, 25, 50 and 100µg/ml of gallic acid solutions with 5.0 ml of Folin-Ciocalteu reagent (diluted tenfold) and 4.0 ml of sodium carbonate solution (75g/l). The absorbance was measured after 30 min at 20°C at 765 nm. Exactly 1ml of the crude and purified *Erythrophleum suaveolens* lectin (1.0 g/100 ml) was mixed separately, with the same reagents as done in construction of calibration curve. After 30 minutes, the absorbance was measured for the determination of total phenolic compound in both the extract separately.

3.2.7.4 Determination of total tannins

The method of AOAC (1984) was adopted, 1ml each of the crude and purified *Erythrophleum suaveolens* lectins were put in a conical flask and 50ml of distilled water was added. This was allowed to stand for one hour and centrifuged at 1000 rpm for 5 minutes at room temperature and filtered using a whatman filter paper. The residue on the filter paper washed with distilled water and the extract was diluted to volume (this is the extract volume). Exactly, 2.5ml of diluted extract (aliquot volume) was pipetted into a 5ml volumetric flask followed by 1ml Folin-Denis reagent and was diluted to volume with distilled water. The solution was allowed to stand for 30minutes at room temperature. The absorbance was read at 700nm and extrapolated on a standard tannic acid curve.

3.2.7.4.1 Preparation of Tannin Standard Calibration Curve

The tannic acid standard solution was prepared by dissolving 0.2g of tannic acid in distilled water and diluted to 200ml mark (1mg/ml). Varying concentration (0.1-1.0 mg/ml) of standard tannic acid solution, exactly 5ml Folin-Denis reagent and 10ml

saturated sodium carbonate ($NaCO_3$) solution were pipette into 6 test tubes and made up to the 100ml mark with distilled water. The absorbance was read after 30 minutes at 700nm. The absorbance was plotted against concentration of tannic acid.

3.2.8 Larvicidal Bioassay

3.2.8.1 Breeding of *Culex quinquefasciatus* Larvae in the Laboratory

The collected *Culex quinquefasciatus* larvae were reared according to (WHO, 1996) standard of breeding mosquitoes in the laboratory. About 500 larvae were placed in a transparent plastic bucket containing about 500ml of water and covered with polystyrene netting, held with a rubber band and kept at room temperature. The larvae were fed with a pinch of ground fish and yeast diet. The set up was monitored daily; the adults that emerged were harvested by suction with an aspirator and transferred into a cage. The adult mosquitoes were fed on a de-haired guinea pig and 20% (w/v) sucrose solution which was soaked in an absorbent cotton wool. The blood fed adult mosquitoes in the cage laid eggs in a bowl of water that was placed in the cage. The eggs hatched within 2 - 3 days producing the first Filial (F1) generation. The first instar larvae were distributed in bowls 30 cm in diameter and 12.5m in depth. Care was taken to prevent over-crowding. The larvae were kept in the plastic bowels half filled with tap water and fed on a pinch of ground fish meal once a day initially and twice during the later stages of development until they developed into the third instar larvae required for the study. The water in rearing container was refreshed every day by removing a little quantity of water from the rearing buckets and replacing with fresh water. This was aimed at preventing scum from forming on the water surface.

3.2.8.2 Bioassay of First Filial (f1) Generation of *Culex quinquefasciatus* Larvae with Crude and Purified *Erythrophleum suaveolens* Lectin

A 1.0% ($^{w}/_{v}$) stock solution each of the crude and purified *Erythrophleum suaveolens* lectins were made and kept separately in a screwed cap vials. First, the stock solutions were serially diluted (at tenfold a time) in distilled water to the 6^{th} dilution to give solutions with the following concentrations: 1.0%, 0.1%, 0.01%, 0.001%, 0.0001% and 0.00001%. From each dilution, 0.1ml, 0.5ml and 1.0ml were transferred to a separate plastic dish containing 25 third-instar larvae of *Culex quinquefasciatus*, in 100ml of distilled water (making a total of 18 plastic dishes). A control dish (without lectins) was setup alongside the experimental ones. The depth of the distilled water in the dishes was between 5cm to 10cm to avoid mortality due to lack of oxygen or water. The larvae in all the bowls were fed every twenty four hours on a pinch of ground fish meal which was spread evenly across the water surface. The larval mortalities were recorded after 24hrs and 48hrs exposure. All the mortalities were counted and recorded as percent corrected mortalities. The experiment was repeated twice to give triplicate values. The LC_{50} and LC_{90}values of the crude and purified bark extract of *Erythrophleum suaveolens* for *Culex quinquefasciatus* were obtained separately by calculating the regression line employing probit analysis of Finney, (1964) as described by Busvin, (1971).

3.3 STATISTICAL ANALYSIS

The corrected percentage mortality was expressed as mean ± SEM and analyzed by ANOVA.

CHAPTER FOUR

RESULTS

4.1 ISOLATION AND PURIFICATION OF LECTINS FROM *Erythrophleum suaveolens* STEM BARK

Lectin from the saline extract of *Erythrophleum suaveolens* stem bark was isolated, a total volume of 500ml of the crude extract was collected, the summary of the purification scheme of lectins from the stem bark of *Erythrophleum suaveolens* was as represented in Table 4.1. The crude extract had higher haemagglutinating activity (HA) of 1.1HU/ml compared to 0.9HU/ml observed in the 20%-90% ammonium sulphate fractionation, 0.7HU/ml observed in Gel I, 0.8HU/ml observed in Gel II, and 0.5HU/ml observed in both Ion I and II fractions respectively. The protein concentration and percentage yield of the crude saline extract was also higher than the ammonium sulphate concentrated fraction, gel filtrated fraction and ion exchange fractions by 44.74mg and 100% respectively.

4.1.1 Gel Filtration Chromatography

The gel filtration chromatography and haemagglutinating activity of the 20%-90% ammonium sulphate fraction of *Erythrophleum suaveolens* was as represented in Figure 4.1. Two peaks were obtained from the elution process, Fractions 14 and 38 with haemagglutinating activity of 0.7HU/ml and 0.8HU/ml respectively with a total protein concentration of 12.59 mg and 14.56 mg, specific activity of 0.06 and 0.05, purification fold of 2 and 1.67 and a recovery efficiency of 1.27% and 1.46% respectivel

Table 4.1: Summary of Purification Steps of Lectins from the Stem Bark of *Erythrophleum suaveolens*

Steps	Total protein (mg)	Total activity (HU)	Specific Activity (Unit/mg)	Purification fold	% yield
Crude extract	22,380	550	0.03	1	100
NH_4SO_4	3176	90	0.03	1	16.36
Gel I	125.95	7	0.06	2	1.27
Gel II	145.6	8	0.05	1.67	1.46
Ion I	1.06	2.5	2.36	78.67	0.46
Ion II	1.5	2.5	1.67	55.67	0.46

Data for 25g of *Erythrophleum suaveolens* stem bark. Volume of the crude extract used was 500ml

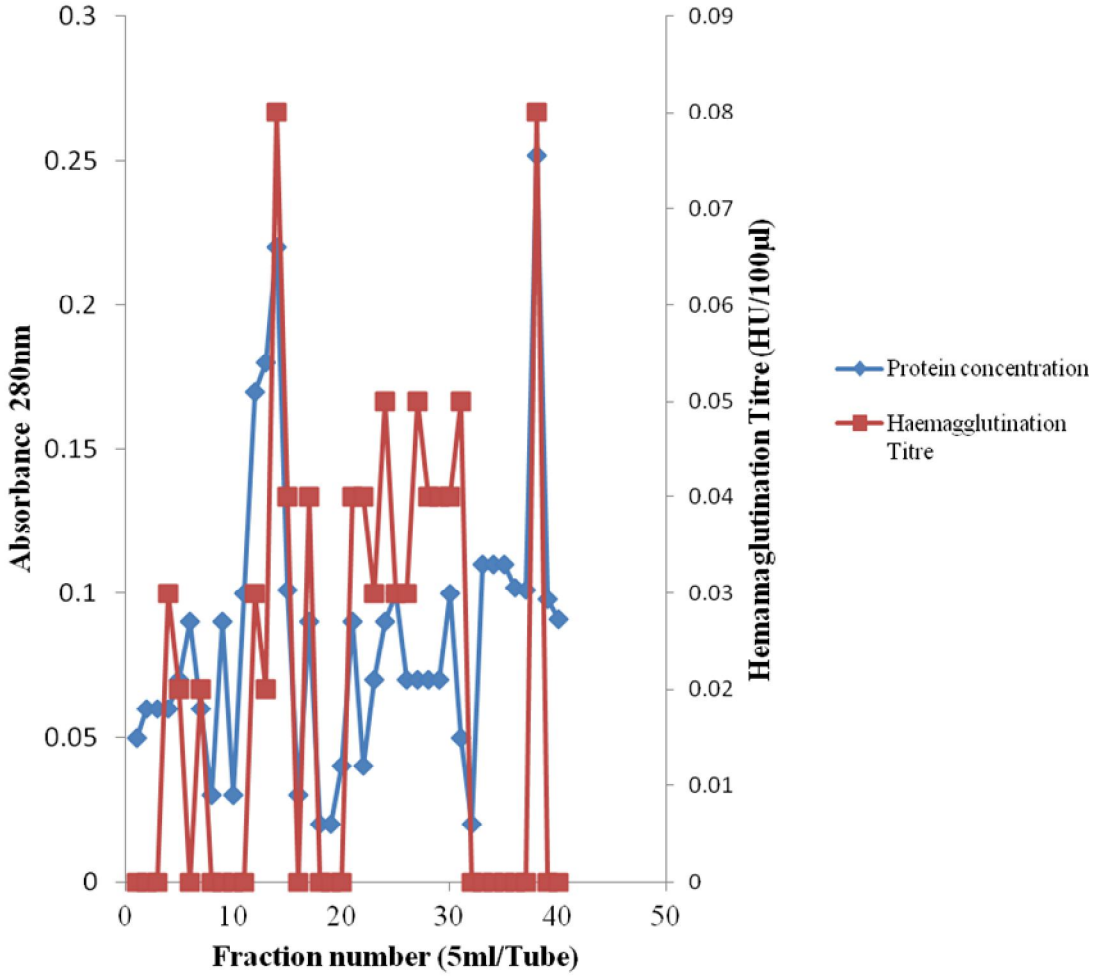


Figure 4.1: Elution Profile and Haemagglutination Activity of 20-90% Ammonium Sulphate Fractions of *Erythrophleum suaveolens* Lectin from Sephadex G-75 Gel Filtration Column

4.1.2 Ion Exchange Chromatography

The elution profile of fraction 14 (Figure 4.1) was as represented in Figure 4.2; a single peak was obtained with haemagglutination activity of 0.5HU/ml and protein concentration of 1.06mg, specific activity of 2.36, and purification fold of 78.67 and a recovery efficiency of 0.46%. While the elution profile of fraction 38 (Figure 4.2) was as represented in Figure 4.3, a single peak was also obtained with haemagglutination activity of 0.5HU/ml and protein concentration of 1.5mg, specific activity of 1.67, and purification fold of 55.67 and a recovery efficiency of 0.46%.

4.1.3 Sodium Dodecyl Sulphate Polyacrylamide-Gel Electrophoresis (SDS PAGE)

The electrophoregram of the crude and purified proteins was as represented in Plate 4.1. The molecular makers are represented in lane 1. The crude lectin showed one band with molecular weight of 38KDa as represented on lane 2. The NH_4SO_4 concentrated protein also showed a single band with molecular weight of 36KDa as represented on lane 3. Gel fractions I and II both showed single bands each with molecular weights of 28KDa, and 26KDa (shown on lanes 4 and 5) respectively. Ion fractions I and II also showed single bands each with molecular weights of 11KDa and 9KDa (shown on lanes 6 and 7) respectively.

4.2 Partial Charaterisation of *Erythrophleum suaveolens* Lectins

4.2.1 Thermostability of Isolated *Erythrophleum suaveolens* Lectins

The effect of temperature stability on haemagglutinating activity was as represented in Figure 4.4. The haemagglutinating activities of the crude and purified lectins were stable within a temperature range of 30°C-60°C. Above this temperature range the haemagglutinating activity of the lectins rapidly falls and was completely lost 90°C-100°C.

4.2.2 Effect of pH on Haemagglutinating Activity of Isolated *Erythrophleum suaveolens* Lectins

The effect of pH on haemagglutination was as represented in Figure 4.5. The haemagglutinating activity of the crude and purified lectins was stable within a pH range of 3-7, the haemagglutinating activity at of 7.4 the haemagglutinating activity dropped rapidly and was completely lost at pH 8-12.

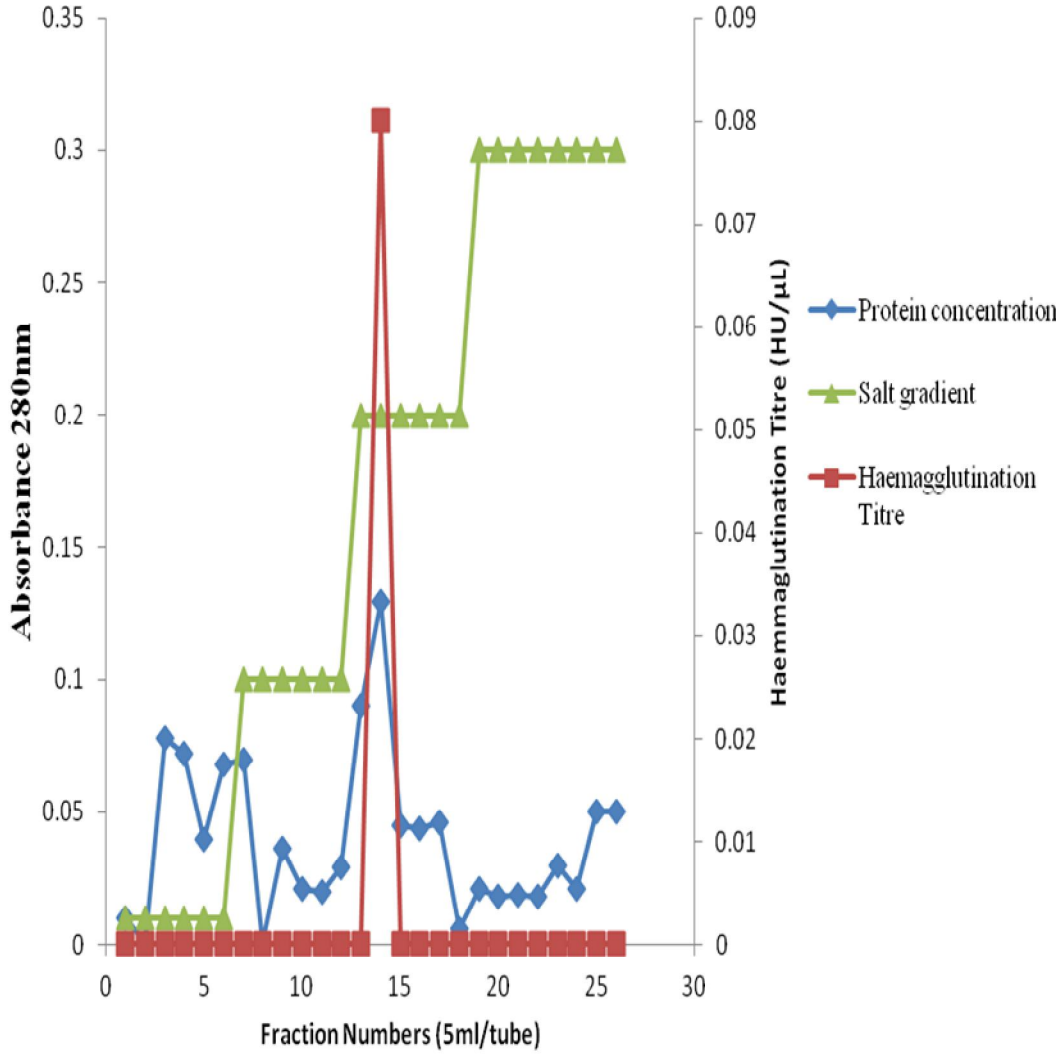


Figure 4.2: Elution Profile of Pooled Ion Exchange Chromatographic Column Fraction I of *Erythrophleum suaveolens* Lectin from Sp- Sephedex C-50

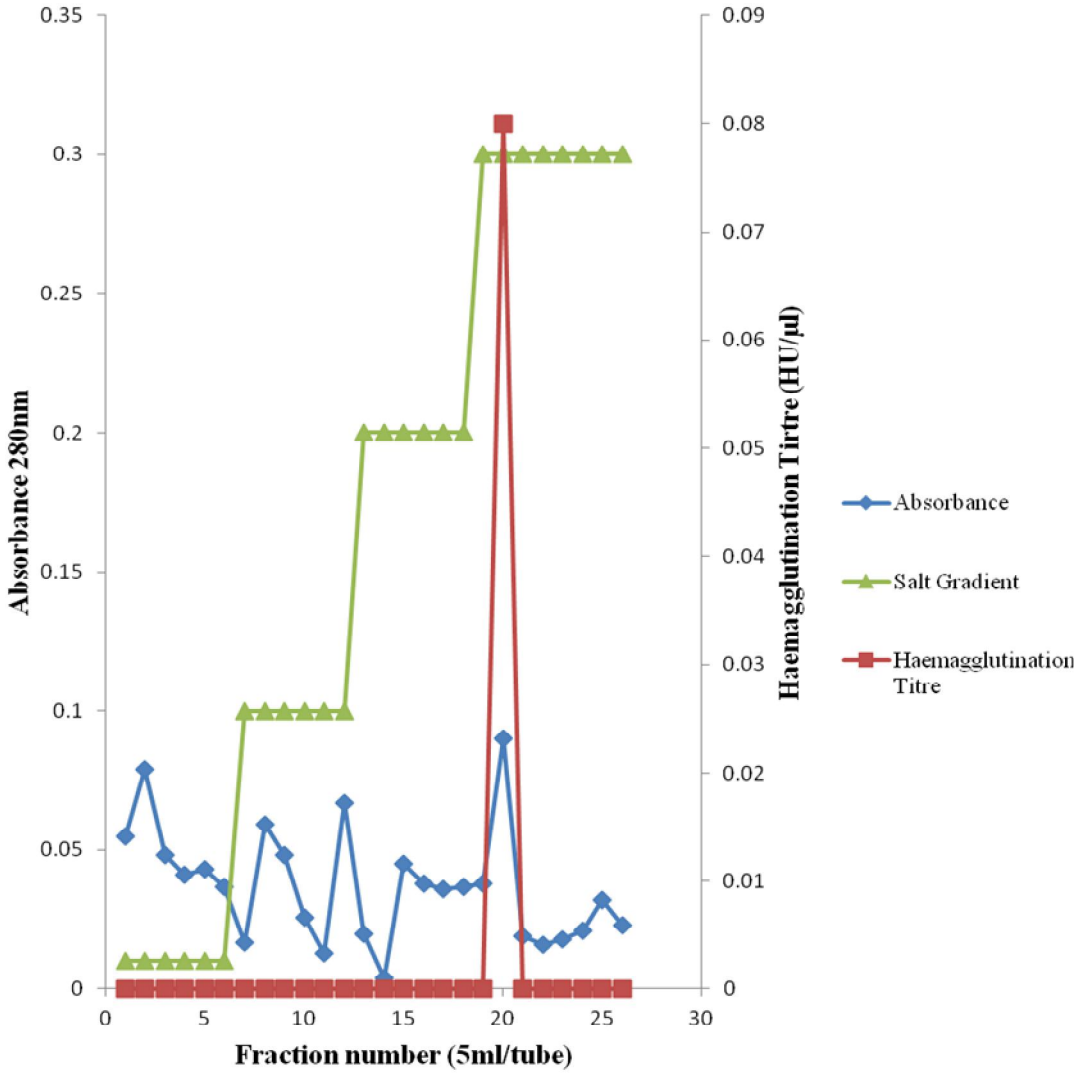


Figure 4.3: Elution Profile of Pooled Ion Exchange Chromatographic Column Fraction II of *Erythrophleum suaveolens* Lectin from Sp- Sephedex C-50

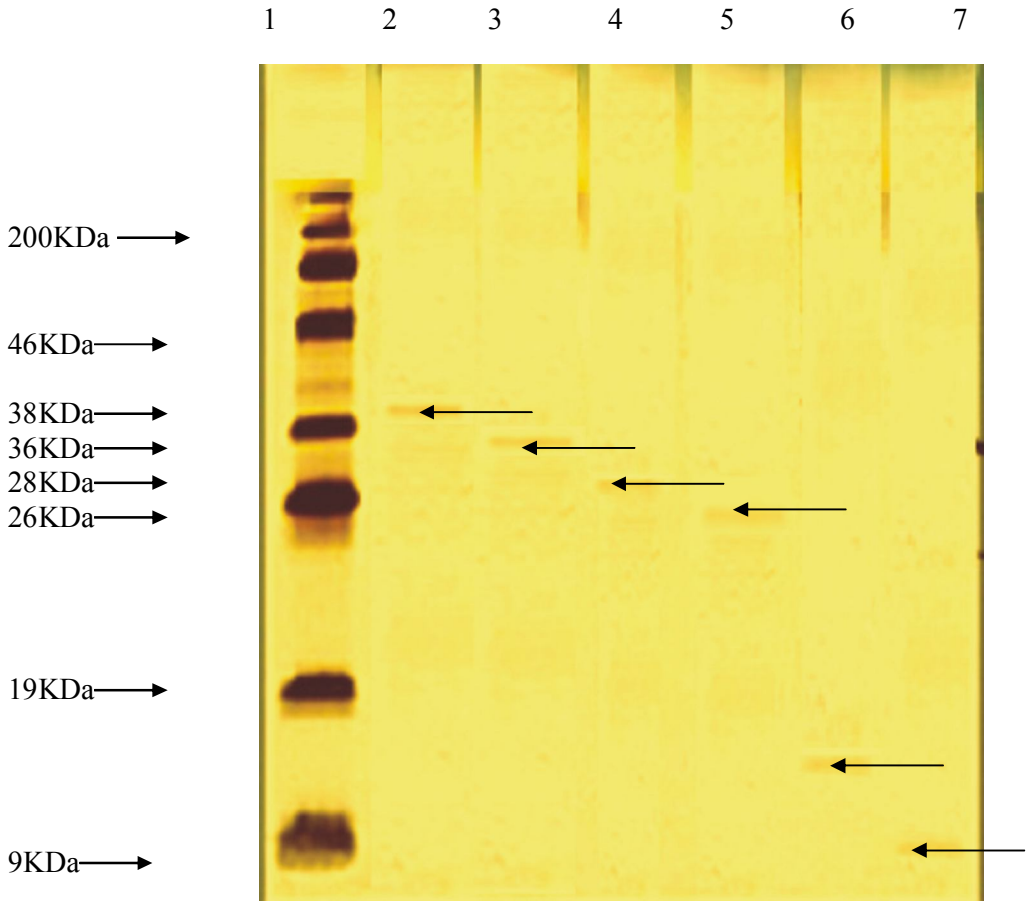


Plate 4.1 Electophoregram of Proteins from the Various Fractions

Lane 1- Molecular Markers
Lane 2- Crude Extract (38KDa)
Lane 3- NH_4SO_4 Concentrate (36KDa)
Lane 4 - Gel I (28KDa)
Lane 5- Gel II (26KDa)
Lane 6- Ion I (11KDa)
Lane 7- Ion II (9KDa)

4.2.3 Sugar Inhibition Assay of Isolated *Erythrophleum suaveolens* Lectin

The sugar specificity assay of the crude and purified *Erythrophleum suaveolens* lectins was as represented in Figure 4.6. Inhibition of heamagglutination was observed with galactose and mannose at 0.5M of the various sugars. The same effect was also observed at 1M of the same sugars as was represented in Figure 4.7.

4.2.4 Total Carbohydrate Content of Isolated *Erythrophleum suaveolens* Lectin

The total carbohydrate content of the lectins was as represented in Table 4.2. The carbohydrate content range from 0.01% - 0.04%.

4.3 Concentration of some Phytochemicals of the Crude and Purified Fractions of *Erythrophleum suaveolens* Lectin

The mean saponins, alkaloids, tannins and phenols concentration was as represented in Table 4.3. The crude extract had significantly higher quantities of saponins (3mg), alkaloids (0.35mg), tannins (0.96mg) and phenols (0.56mg) than gel peaks I, II, Ions I and II fractions. Gels I and II fractions have generally similar contents of phytochemicals. Ions I and II also do not have significantly different levels of phytochemicals.

4.4 LARVICIDAL BIOASSAY

The relationship between larvae mortality and the concentration of the crude and purified fractions after 24 hours was as represented in Figure 4.8. The corrected percentage mortality for the larvicidal activity of the crude extract of *Erythrophleum suaveolens* bark produced 88% death at 100mg/L concentration. At 50 mg/L concentration the mortality recorded was 86% and at 10mg/L concentrations the percentage mortality recorded was 80%. The same relationship monitored after 48 hours is as represented in Figure 4.9. Increase of exposure time to 48hrs raised the percentage mortality of the crude *Erythrophleum suaveolens* lectin to 96% at 100mg/L concentration. At 50 mg/L and 10mg/L concentrations the mortalities recorded were similar (93.33%). The Gel peak I

fraction produced, 68% and 74.67% mortalities at 100mg/L concentration after 24 and 48 hours exposure respectively. While at 50 mg/L concentration the mortalities recorded were 64% and 68% after 24 and 48 hours exposure respectively. At 10mg/L concentrations the percentage mortalities recorded were 58.67% and 62.67 % after 24 and 48 hours exposure respectively. Gel peak II fraction resulted in 65.33% and 69.33% mortalities at 100mg/L concentration after 24 and 48 hours exposure respectively. At 50 mg/L concentration the mortalities recorded were 60% and 65% after 24 and 48 hours exposure respectively. At 10mg/L concentration the mortalities recorded were 49.33% and 60% after 24 and 48 hours exposure respectively. Ion peak I fraction produced similar mortalities (10.67%) at 100mg/L concentration after 24 and 48 hours exposure respectively. At 50 mg/L concentration the mortalities recorded were 0% and 1.33 % after 24 hours and 48 hours exposure respectively. At 10mg/L concentrations there was no mortality recorded after 24 and 48 hours exposure. The Ion peak II fraction resulted in 4 % and 9.33% mortalities at 100mg/L concentration after 24 hours and 48 hours exposure respectively. At 50 mg/L concentration the mortalities recorded were 0% and 9.33% after 24 and 48 hours exposure respectively. At 10mg/L concentrations there was 1.33% mortality was recorded after 24 hours exposure while 4% mortality was recorded after 48 hours exposure. Based on probit analysis the crude extract had LC_{50} of 4.49 mg/L and LC_{90} of 16.20mg/L; while the gel peaks I and II fractions had LC_{50} and LC_{90} of 10mg/L and 43.73 mg/L respectively.

The larvicidal activity of the extract reduced with purification, one would think that the activity would have increased as the extract gets purer, this may have been as a result of some human errors.

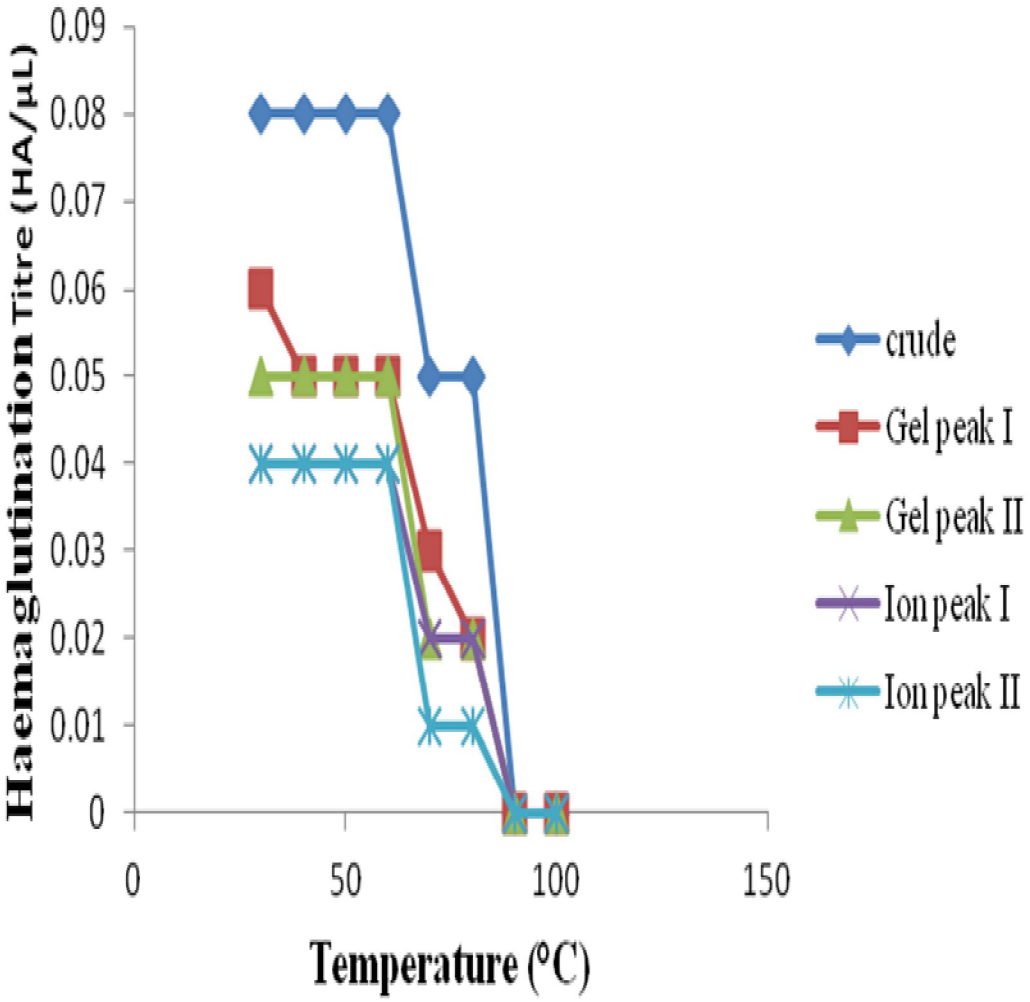


Figure 4.4: Effect of Temperature on Haemagglutinating Activity of Crude and Purified *Erythrophleum suaveolens* Lectins

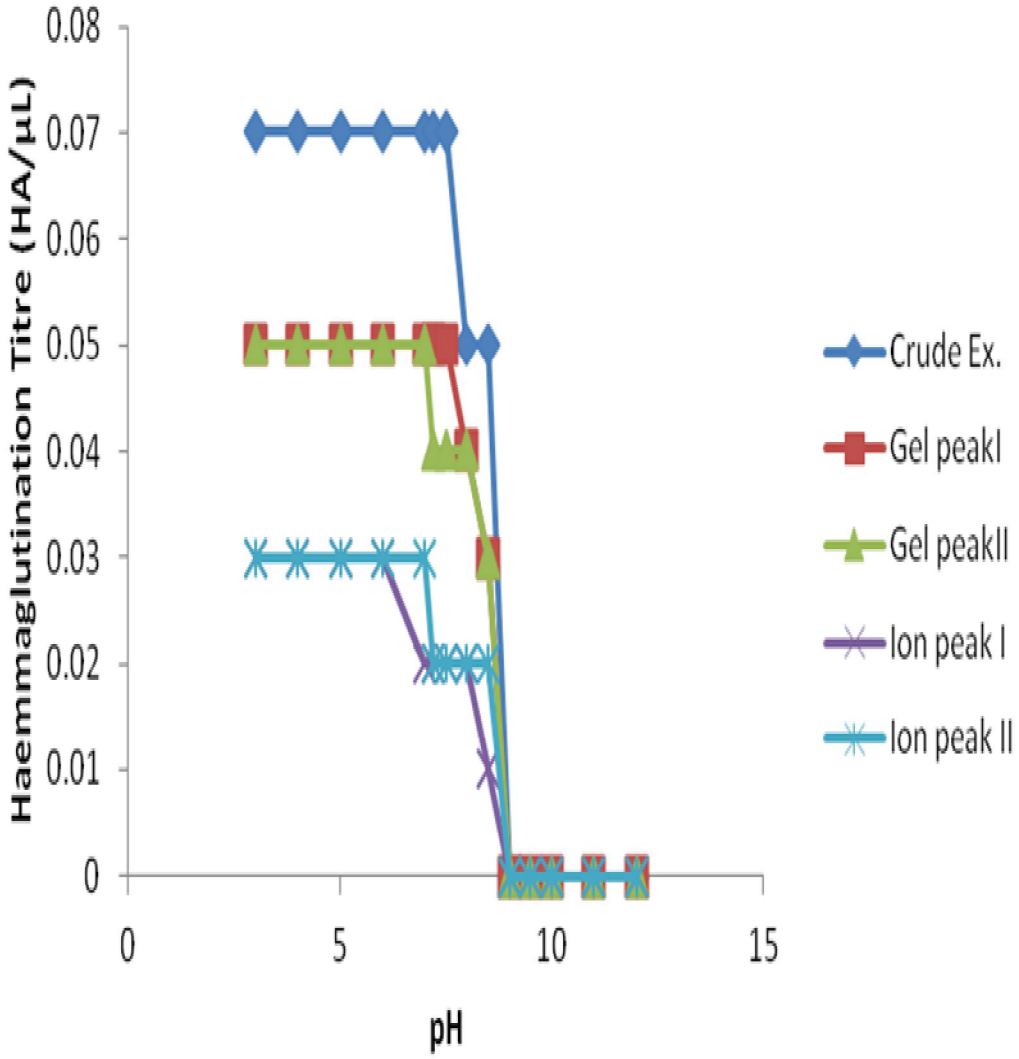


Figure 4.5: Effect of pH on Haemagglutinating Activity on Crude and Purified *Erythrophleum suaveolens* Lectin

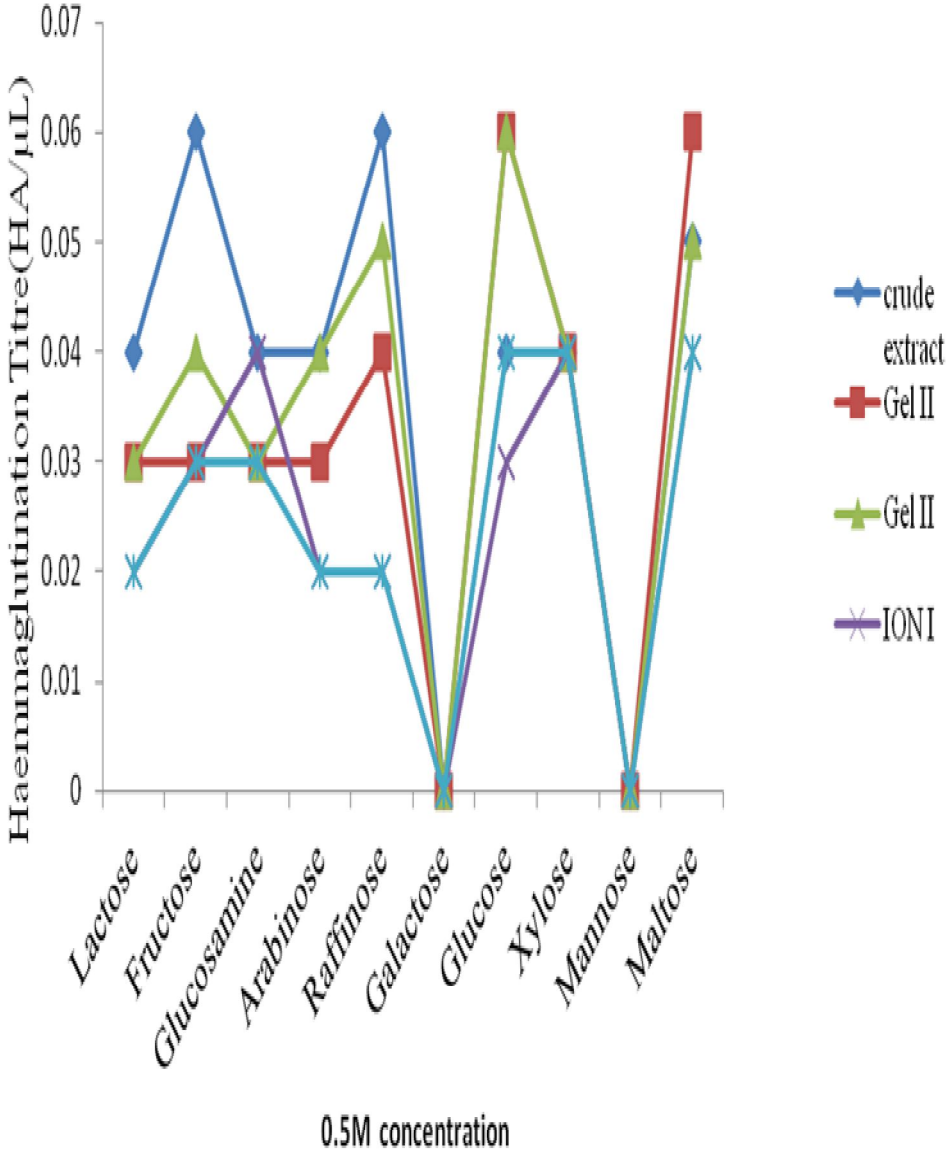


Figure 4.6: Haemaglutinating Activity of *Erythrophelum suaveolens* Lectins in the Presence of Different Sugars at 0.5M Concentration

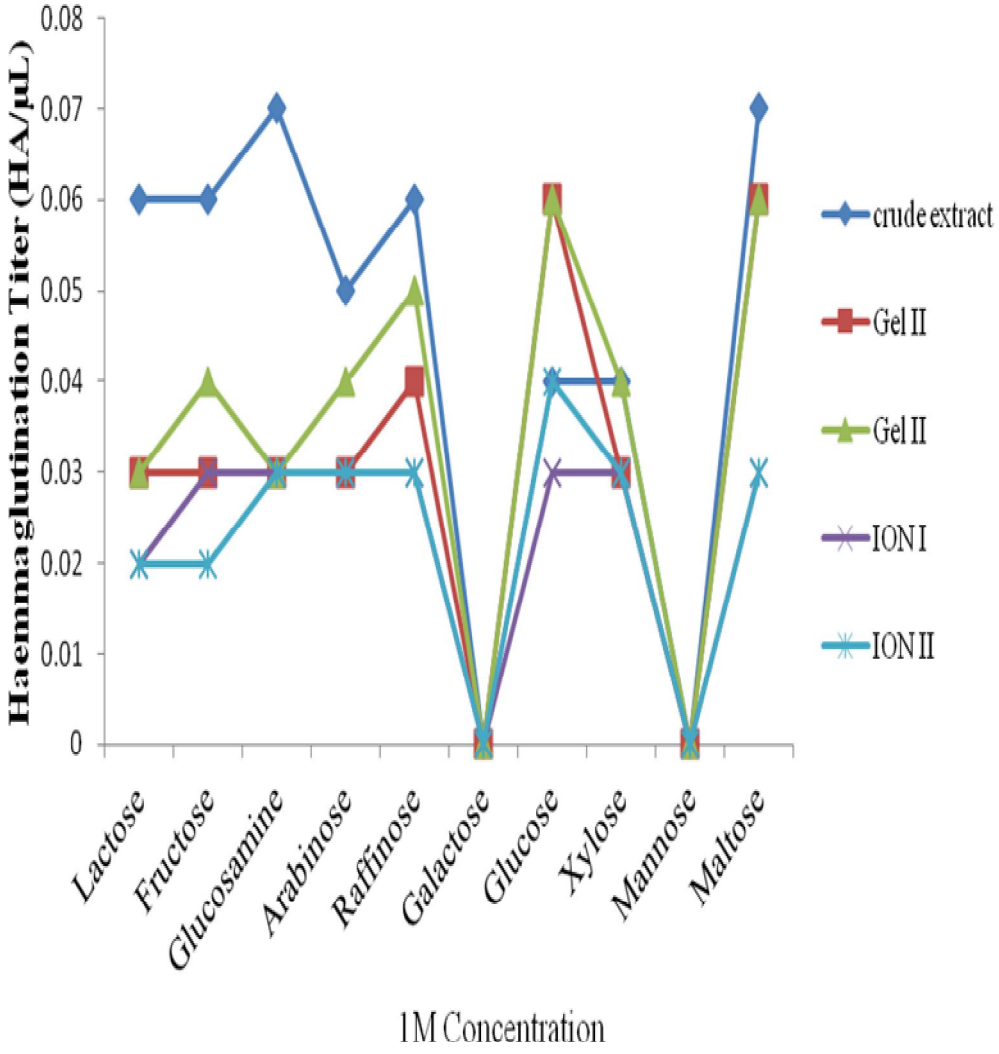


Figure 4.7: Haemaglutinating Activity of *Erythrophelum suaveolens* Lectins in the Presence of Different Sugars at 1M Concentration

Table 4.2: Total Carbohydrate Contents of Crude and Purified *Erythrophleum suaveolens* Lectin Fractions

Sample	% Carbohydrate Content
Crude Extract	0.04
Gel peak I	0.04
Gel peak II	0.03
Ion peak I	0.02
Ion peak II	0.01

Table 4.3: Levels (mg/100g) of Some Phytochemicals in the crude and Purified *Erythrophleum suaveolens*

Phytochemicals	Crude Extract	Gel I	Gel II	Ion I	Ion II
Saponin	3.00^{c}±0.26	1.50^{b} ±0.01	1.53^{b}±0.03	0.50^{a}±0.02	0.35^{a}±0.01
Alkaloid	0.35^{c}±0.02	0.05^{b}±0.03	0.02^{ab}±0.03	0.01^{a}±0.01	0.02^{ab}±0.01
Tannin	0.96^{e}±0.02	0.50^{d}±0.02	0.40^{c}±0.04	0.10^{a}±0.03	0.15^{b}±0.01
Phenol	0.56^{e}±0.03	0.36^{c}±0.02	0.22^{b}±0.01	0.12^{a}±0.03	0.18^{b}±0.03

Each value represents the mean ± SD from triplicate determinations.
Values on the same row with different superscripts are significantly different at $p< 0.05$.
Values on the same row with the same superscript are significantly similar at $p<0.05$.

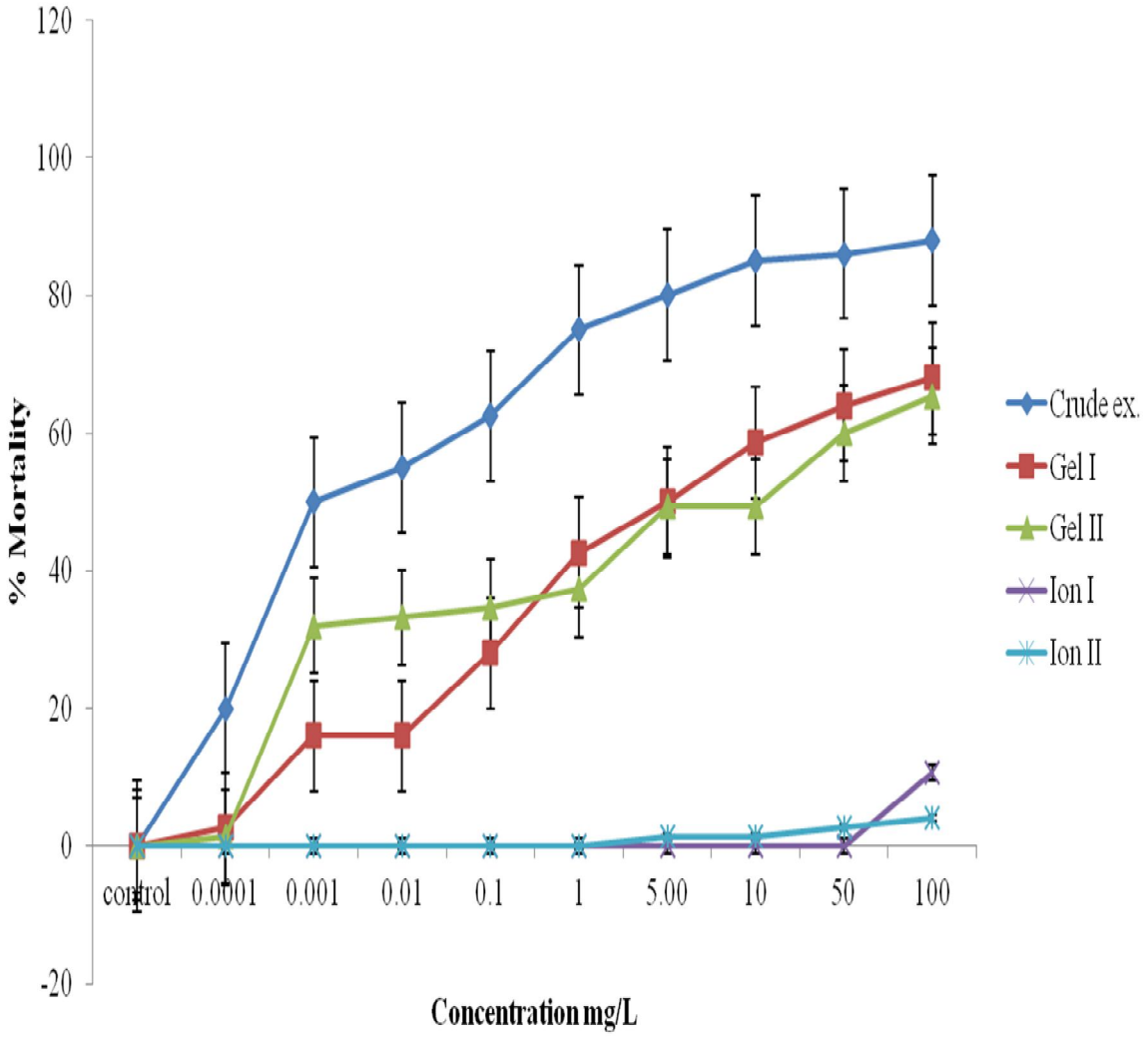


Figure 4.8: The Corrected Percentage Mortality (± SEM) of Third Instar Larvae of *Culex quinquefasciatus* Exposed to Different Concentrations of Crude and Purified *Erythrophleum suaveolens* Lectin After 24hrs Exposure

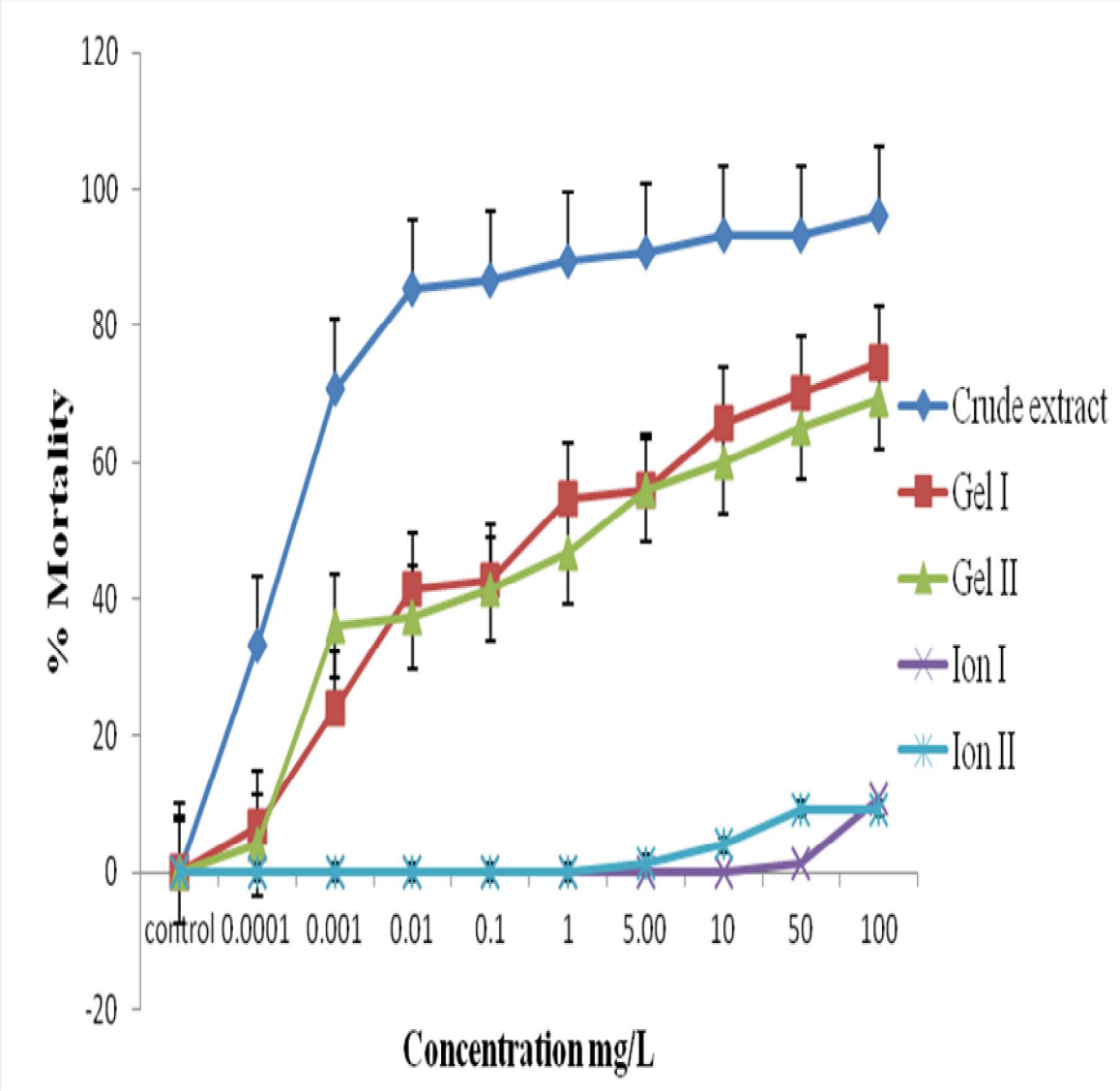


Figure 4.9: The Corrected Percentage Mortality (± SEM) of Third Instar Larvae of *Culex quinquefasciatus* Exposed to Different Concentrations of Crude and Purified *Erythrophleum suaveolens* Lectin After 48hrs exposure.

Table 4.4: Log probit Regression Analysis of the Mortality of 3rd Instar Larvae of *Culex quinquefasciatus* to Crude and Purified *Erythrophleum suaveolens* Lectin

Samples	Regression Equation[c]	Chi-Square[b] (DF)	[a]LC_{50} (Fiducial Limit)	[a]LC_{90} (Fiducial Limit)
Crude ex.				
100mg/l	y= 2.33x+3.5	0.42 (4)	4.49 -	16.20 -
50mg/l	y= 2.33x+3.5	2.02 (4)	4.49 (-14.90-2.23)	16.20 (-56.782-10.354)
10mg/l	y= 2x+3	0.26 (4)	10 (-1.90-4.33)	43.73 (-61.16-7.35)
Gel1				
100mg/l	y= 2x+3	0.36 (3)	10 (-1.80 -0.48)	43.73 (-17.76-4.40)
50mg/l	y= 2x+3	2.71 (2)	10 (-0.50-1.28)	43.73 (-2.926-0.857)
10mg/l	y= 1.67x+2.5	2.02 (1)	29.55 (0.56-3.54)	167.68 (-18.41-1.05)
Gel2				
100mg/l	y= 2x+3	4.73 (4)	10 (-2.00-0.73)	43.73 (-25.88-6.21)
50mg/l	y= 2x+3	2.02 (1)	10 (0.556-3.54)	43.73 (-18.41-1.05)
10mg/l	y= 1.67x+2.5	59.91 (3)	29.55 -	167.68 -
Ion1	y= 0	0	0	0
Ion2	y= 0	0	0	0

[a] Lethal concentrations of proteins required to kill 50% (LC50), 90% (LC90) the 3rd instar larvae of *Culex quinquefasciatus* after 48hours exposure.
[b] chi-square values.
[c] Simple linear regression and regression coefficient values (R2) established using by probit analysis.
y: mortality rate (%);
x: protein concentration (mg/mL).

CHAPTER FIVE

DISCUSSION

5.1 ISOLATION AND PURIFICATION OF LECTIN FROM *Erythrophleum Suaveolens* STEM BARK

Initially, purification of lectins followed the scheme used for proteins in general without exploiting their special characteristics. The methods used included precipitation by salts, acids and organic solvents. Progress in this area was achieved by the introduction of preparative chromatographic methods with ion exchangers, gel filtration (size exclusion chromatography) media and, above all, affinity adsorbents (Rüdiger and Gabius, 2001). In this work, lectins were isolated and partially purified from the saline extract of *Erythrophleum suaveolens* stem bark. The precipitation of the lectin was done with ammonium sulphate. Approximately 16% of the proteins present in the crude *Manihot esculenta* extract could be recovered following precipitation in 20-90% ammonium sulphate. The recovery efficiency obtained in the present study was greater than that reported previously (11%; Pereira *et al.,* 2008). The concentrated ammonium sulfate precipitate of *Erythrophleum suaveolens* lectin subjected to gel filtration chromatography showed two peaks. This may be an indication that the lectin is large and has a high molecular weight since it was eluted first at fraction 14.The fractions with the highest haemagglutinating activity from the first step subjected to ion exchange chromatography seperately showed two peaks.

Elution of proteins bound to a stationary phase can be achieved by introducing a soluble ligand that competes with the immobilised ligand to which the target protein is attached (Collins *et al.,* 1997). In the case of lectins, the linkage with a soluble ligand (e.g.galactose) could occur either through the carbohydrate receptor site (specific linkage) or through ionic, hydrophobic and hydrogen interactions (non-specific linkage). In the present study, the Sp -sephadex column was eluted with 0.01 - 0.3M NaCl in an attempt

to elute the bound protein was sufficient to break the weak non-specific interactions, thus displacing the bound lectin from the stationary phase fraction. Maria *et al.,* 2010, reported that a Sepharose column was eluted with 0.15 M NaCl containing 0.1 M galactose in to elute the bound protein fraction, but none of the eluted fractions showed absorption at 280 nm. The carbohydrate receptor site of the *Manihot esculenta* lectin is not specific for the galactose ligand, this is based on the fact that the agarose gel of the stationary phase is highly cross-linked forming pores of different sizes such that the proteins were separated according to molecular weight.

The crude and purified lectins moved as a single band with different mobilities on SDS-PAGE electrophoregram. Evaluation of *Myracrodruon urundeuva* bark lectin and *Myracrodruon urundeuva* hardwood lectin by electrophoresis showed single polypeptide bands in PAGE as reported by (Roberto *et al.,* 2009)**.** Concanavalin isolated from *Canavalia ensiformis* by Sheldon *et al* (1998) yielded five bands of molecular weights 78, 74, 54, 32 and 30 kDa on SDS PAGE. Electrophoresis under native conditions separate proteins according to molecule net charges and is able to evaluate the homogeneity of protein (Monti *et al*., 2008). The detection of single band only in PAGE for basic proteins revealed that both lectins have positive net charge and that the established purification protocols yielded homogeneous lectins (Roberto *et al.,* 2009)**.**

5.2 CHARACTERIZATION OF CRUDE AND PURIFIED LECTIN OF *Erythrophleum suaveolens*

Inhibition occurred with mannose and galactose, indicates that the sugar-lectin interaction was stronger than the erythrocyte-lectin attraction and consequently formed a distinct button of the erythrocytes at the bottom of the well. Therefore, *Erythrophleum suaveolens* lectin is mannose and galactose sugar specific. Bourne *et al.*, (2002) reported that carbohydrate binding specificity was exhibited by jacalin, the seed lectin from

Artocarpus integrifolia towards galactose and mannose. Structural analysis demonstrated that this may be due to the large size of the carbohydrate-binding site of jacalin that enables it to accommodate monosaccharides with different hydroxyl conformations (Laija *et al.,* 2010).

Carbohydrates binding, particularly mono and disaccharides, is the defining feature of the lectins and is the basis of many methods of classification of plant lectins (Laija *et al.,* 2010). Lectins from *Artocarpus* species (jacalin and artocarpin) were classified as T-specific lectins (Wu and Shunji, 1991). The saccharide binding specificities of lectins can be utilized in the purification, characterization and sequencing of polysaccharides, polypeptides and glycoproteins (Laija *et al.,* 2010). Peumans *et al.,* (2000a) reported inhibition by monosaccharides on lectins like galactose and mannose on Jacalin, mannose on arthocarpin (Barre *et al., 2004*). Galactose on *Aphytis lignanensis* lectin (Zhang *et al.,* 1999). Complex sugar like N-acetyl-D-glucosamine inhibited artocarpin (Mourao *et al.,* 1999) and *Aphytis lignanensis* lectin (Zhang *et al.,* 1999). While *Artocarpus hirsute* lectin is inhibited by methyl-α-D-galacto-pyranoside, p-nitro-phenyl (pNp) - α-D-galacto-pyranoside and pNP- α-D-N-acetyl-galactosamine (Gurjar *et al.,* 1998). The other sugars (glucosamine, glucose, maltose, lactose, arabinose raffinose, maltose and xylose) showed agglutination to *Erythrophleum suaveolens* lectins. This indicates that the lectin contains numerous binding sites that were compatible with a wide range of monosaccharides receptors. Non-inhibition of agglutination by some of the sugar molecules indicates that erythrocyte-lectin interaction may be due to affinity for lectin substitution at certain positions of the sugar moiety which determines their specificity (Lacsamana and Merca, 1994). It could also be as a result of the non specificity of lectin binding sites to the individual sugar anomers (Lacsamana and Merca, 1994). The erythrocytes-lectin binding may not be affected by the size and shape of sugar residues (Lacsamana and Merca,

1994). The carbohydrate containing the sugar or its derivatives may cross react with the lectin (Lacsamana and Merca, 1994).

The crude and purified lectins of *Erythrophleum suaveolens* was characterized by fair thermostability probably due to disulfide bonds or other factors. It is stable up to 60 ◦C compared to *Tricholoma mongolicum* lectin is stable between 10 and 80^{o}C. *Hevea brasiliensis* lectin was reported, to have a stable haemagglutinating activity at 60^{o}C (Wittsuwannakul *et al*., 1998) while *Ptilota filicina lectin* was reported to be stable at 50^{o}C (Sampaio *et al*., 1998). Stability over a wide temperature range is typical of plant lectins and can be attributed to their role in plant defense mechanisms against environmental stress (Peumans and Van Damme, 1995). The stability of lectins from *Erythrophleum suaveolens*, similar to other plant lectins was probably dependent upon several structural characteristics. The compact globular structures of lectins due to high number of hydrophobic interactions, hydrogen bonds and disulphide bridges (intra and inter chains), molecular aggregation and glycosylation in general results in high structural stability of native lectins (Moreno *et al.,* 2008; Kawsar *et al.,* 2008). sThe crude and purified lectins of *Erythrophleum suaveolens* are glycoproteins in nature because they gave orange yellow color in the presence of phenol-sulfuric acid with different carbohydrate contents which is in accordance with the report of Khan *et al.,* (2008) on *Chorchorus olitorius* lectins which contained 1.3%, 1.2% and 0.8% neutral sugars, Syed *et al*., (2001) also reported the formation of orange yellow color in the presence of phenol-sulfuric acid by rice-bran lectin with sugar and neutral content of 4% and 5.4% Kuku and Eratan (2004) reported that a purplish pink colour was obtained by staining *Kalanchoe crenata* lectin with Schiffs reagent.

The crude and purified *Erythrophleum suaveolens* lectin had a stable haemagglutination activity within the pH range of 3-7. The activity, however, fell off fairly rapidly

thereafter, with essentially all activity lost after pH 9.2 as represented in Figure 4.5. It is possible that changes in the ionization state with an increase in pH may have led to a weaker binding of the metal ions, which were apparently required for the maintenance of the structure that was required for maximal activity Kuku and Eretan (2004). The pH dependence, which is observed in virtually all enzyme reactions, is a consequence of the protein composition Kuku and Eretan (2004). Numerous ionisable groups at the surface of the protein molecule and the active center are capable of reacting with H^+ or OH^- Kuku and Eretan (2004). Any pH change is therefore associated with a change in the ionization state of the molecule, which in turn, determines the binding forces between enzyme and substrate (Adolph and Lorenz, 1982). It is also possible that the increase in OH− ions causes a change in the ionization state of the lectin, thereby affecting the binding forces between the lectin and the erythrocyte membrane that eventually lead to a loss of activity. The lectin of *Pitilota filicina* was also stable in the pH range of 4-9, retaining 50% of its activity at pH 3 and 10, and 25% of its activity at pH 11-12 (Sampaio *et al.*, 1998). The lectin from *Parkia javanica* beans was stable in the pH range of 7-10 (Utarabhand and Akkayanont, 1995). It was also reported that, more acidic or basic pH decreases both the stability and activity of lectins (Utarabhand and Akkayanont, 1995).

5.3 PHYTOCHEMICAL COMPOSITION OF CRUDE AND PURIFIED *Erythrophleum suaveolens* Lectins

The crude extract had higher tannins, phenols, saponins and alkaloids compared to the purified fractions. Similarly the phytochemical analysis of the *Erythrophleum suaveolens* stem bark extract repoted by Aiyegoro *et al.,* (2007) revealed the presence of saponins, tannins, steroids and alkaloid. Although, some phytochemicals have be attributed to have larvicidal effect on insects, however, for the purpose of this study the quantities of phytochemicals obtained are so minute or almost negligible compared to the quantity of lectins isolated.

5.4 LARVICIDAL EFFICACY

The crude and purified *Erythrophleum suaveolens* lectin preparations were able to kill the 3rd instar larvae of *Culex quinquefasciatus* after 24 and 48hours exposure. The LC_{50} and LC_{90} revealed that purification steps yielded preparations with higher values than the initial crude preparations. The crude fraction gave LC_{50} and LC_{90} values lower than the values obtained for the purified fractions revealing that the lectin activity was lost with further purification.

Roberto *et al.,* (2009) reported that the evaluation of larvicidal activities of lectins from *Myracrodruon urundeuva* on *Aedes aegypti* revealed that purification protocols yielded preparations with lower LC values than initial crude preparations. Since LC_{50} is a standard measure of the toxicity of a surrounding medium that is required to kill half of a sample population, it does account for chronic effects. A lower LC_{50} means that a substance is more toxic and would require less of the substance to kill the organism ingesting it. The varying susceptibility observed here is in line with reports from previous findings that mosquito species showed differential susceptibility to plant extracts (Pathak *et al.,* 2000).

The binding of lectins specific to N-acetylglucosamine to the peritrophic matrix interferes with the digestion and absorption of nutrients (Peumans and Van Damme, 1995; Zhu Salzman *et al.,* 1998; Zhu-Salzman and Salzman, 2001; Carlini and Grossi-de-Sá, 2002; Macedo *et al.,* 2004; Macedo *et al*., 2007). The deleterious effect of lectin on the metabolic processes in the larvae leads to death of the larvae by nutritional deprivation (Fitches and Gatehouse, 1998). The integrity of peritrophic matrix, which has important roles in the digestive processes of insects as well as protection of the insect from invasion by micro-organisms and parasites, is essential for larvae survival (Tellam *et al.,* 1999). Latex of *Calotropis procera* promoted mortality (100%) of third instar larvae of *Aedes*

aegypti and it has been suggested that the toxic effect is at least due to latex proteins (Ramos *et al.,* 2006). Ethanolic extract of *Melia azedarach* leaves showed a strong larvicidal activity with LC_{50} of 0.76 g/L determined after 96 hours of bioassay (Coria *et al.,* 2008). Studies with saponins from *Balanites aegyptiaca* callus produced from *in vitro* cultures of roots revealed that concentrations of 500 ppm or greater killed 100% of the larvae population. Therefore the plant could be used as a larvicidal agent against mosquito (Chapagain *et al.,* 2008). Essential oils from plants have investigated for their insecticidal activity. Heartwoods from *Cryptomeria japonica, Cunninghamia lanceolata, Taiwania cryptomerioide* and *Calocedrus formosana* were sources of larvicidal essential oils with LC50 (µg/mL) of 72.0, 106.4, 79.8 and 75.2, respectively (Cheng *et al.,* 2003). From the above study *Erythrophleum suaveolens* lectin (LC_{50} 4.49 mg/mL or 449 ppm) is more efficient than these heartwood oils mentioned above, and it is lower than that obtained for essential oils from *Hyptis pectinata* (502 ppm) revealing its more strong larvicidal activity (Silva *et al.,* 2008). Cavalcanti *et al*., (2004) reported LC_{50} values between 60 and 538 ppm for 9 essential oils from branches, leaves or fruits of 9 plants of the *Graminae (Poaceae), Labiateae (Lamiaceae), Myrtaceae, Rutaceae, Verbenaceae and Zingiberaceae* families.

The crude extract of *Erythrophleum suaveolens* bark was found to be more lethal to the larvae, followed by gel peaks I and II, while Ion peaks I and II produced the lowest mortality. This might be as a result of loss of activity due to the purification processes employed, resulting is low protein content and hence low activity of the lectin.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 SUMMARY

In this work, lectins were successfully isolated, partially purified and partially characterized from *Erythrophleum suaveolens*. The haemagglutination activity of the crude extract was 0.11HU/µL and the two peaks obtained from the gel filtration had haemagglutinating activities of 0.07HU/µL and 0.09HU/µL and a total protein content of 12.59mg and 14.56mg, giving a recovery efficiency of 1.27% and 1.46% respectively. While the haemagglutination activity from the ion exchange chromatography was 0.05HU/100µl and total protein content of these fractions were 1.06mg and 1.5mg respectively, giving a recovery efficiency of 0.46%. The crude and purified lectins showed single bands each. The purified ion exchange fraction with the highest haemagglutination activity had a protein band at 9KDa. The hemmaglutination activity of the crude and purified *Erythrophleum suaveolens* lectins were stable at temperatures up to 60°C with optimal pH 3-7. The *Erythrophleum suaveolens* lectin is mannose and galactose specific. The results from the study showed that the crude extract and Gel peak I and II fraction exhibited good larvicidal activities on exposure to *Culex quinquefasciatus* mosquito larvae. The lowest mortality was observed in the Ion exchange peaks I and II fraction.

6.2 CONCLUSION

The LC_{50} values of *Erythrophleum suaveolens* demonstrated its good larvicidal activity on *Culex quinquefasciatus*. The most superficial location of the bark in the anatomy of wood gives an advantage because it is easier and safer to the plant to obtain bark tissue for extraction of the lectin. The detection of larvicidal activity in wood resistant to insect degradation provides a significant link between plant physiology and biotechnology.

Therefore, the plant *Erythrophleum suaveolens*, which known for its several uses culturally and in wood utilization contains lectins against *Culex quinquefasciatus* larvae indicating its potential for use as organic insecticide in control of mosquito borne-diseases by disruption of biological cycle of the vector. The crude extract is more effective than the purified fractions, hence, the control of mosquito-borne diseases can be achieved either by causing larval mortality on a large scale at the breeding centers of the vectors in the environment or *Erythrophleum suaveolens* lectin extract can be sprayed in stagnant water bodies and breeding grounds for mosquitoes.

6.3 RECOMMENDATION

This work is not exhaustive, the work is still open for more findings, further work is therefore recommended as follows:

1. Another purification technique that will not interfere with the lectin activity should be employed.
2. Stem of other *Erythrophleum suaveolens* species should be used in future work in order to compare activities with the stem bark used here. This is to determine the effect of senescing, climatic conditions, specie difference, and other factors on lectin activity.
3. The toxicity of *Erythrophleum suaveolens* lectin on aquatic environment should be evaluated in order to estimate its toxic effect on the environment.
4. *Erythrophleum suaveolens* lectins should be tested on the newly discovered dangerous breed of mosquitoes in Kenya.

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APPENDIX 1

Table showing the hemmaglutination titre of fractions collected after gel filtration

Fraction	Absorbance	Protein conc	Hemmaglutination titre	Specific activity
1	0.05	2.89	0	0
2	0.06	3.47	0	0
3	0.06	3.47	0	0
4	0.06	3.47	0.03	0.008
5	0.07	4.05	0.02	0.005
6	0.09	5.20	0	0
7	0.06	3.46	0.02	0.006
8	0.03	1.73	0	0
9	0.09	5.20	0	0
10	0.03	1.73	0	0
11	0.10	5.78	0	0
12	0.17	9.83	0.03	0.003
13	0.18	10.40	0.02	0.001
14	0.218	12.60	0.07	0.008
15	0.10	5.84	0.04	0.01
16	0.03	1.73	0	0
17	0.09	5.20	0.04	0.008
18	0.02	1.16	0	0
19	0.02	1.16	0	0
20	0.04	2.31	0	0
21	0.09	5.20	0.04	0.008
22	0.04	2.31	0.04	0.017
23	0.07	4.05	0.03	0.007
24	0.09	5.20	0.06	0.012
25	0.1	5.78	0.03	0.005
26	0.07	4.05	0.03	0.007
27	0.07	4.05	0.04	0.010
28	0.07	4.05	0.06	0.015
29	0.07	4.05	0.04	0.010
30	0.10	5.78	0.04	0.007
31	0.05	2.89	0.06	0.021
32	0.02	1.16	0	0
33	0.11	6.36	0	0
34	0.11	6.36	0	0
35	0.11	6.36	0	0
36	0.10	5.90	0	0

37	0.10	5.84	0	0
38	0.252	14.56	0.08	0.006
39	0.10	5.66	0	0
40	0.09	5.26	0	0

APPENDIX 2

Table showing the hemmaglutination titre, total protein and specific activity various fractions collected after ion exchange chromatography (I).

Fraction	Absorbance 280nm	Hemmaglutination titre	Salt gradient	Total protein	Specific activity
1	0.010	0	0.01	0.578	0
2	0.001	0	0.01	0.058	0
3	0.078	0	0.01	4.509	0
4	0.072	0	0.01	4.162	0
5	0.04	0	0.01	2.312	0
6	0.068	0	0.01	3.931	0
7	0.070	0	0.1	4.046	0
8	0.001	0	0.1	0.058	0
9	0.036	0	0.1	2.080	0
10	0.021	0	0.1	1.214	0
11	0.020	0	0.1	1.156	0
12	0.029	0	0.1	1.676	0
13	0.090	0	0.2	5.202	0
14	0.1298	0.08	0.2	7.503	0.01
15	0.045	0	0.2	2.601	0
16	0.044	0	0.2	2.543	0
17	0.046	0	0.2	2.66	0
18	0.006	0	0.2	0.35	0
19	0.021	0	0.3	1.214	0
20	0.018	0	0.3	1.041	0
21	0.019	0	0.3	1.098	0
22	0.018	0	0.3	1.041	0
23	0.03	0	0.3	1.734	0
24	0.021	0	0.3	1.214	0
25	0.050	0	0.3	2.890	0
26	0.050	0	0.3	2.890	0

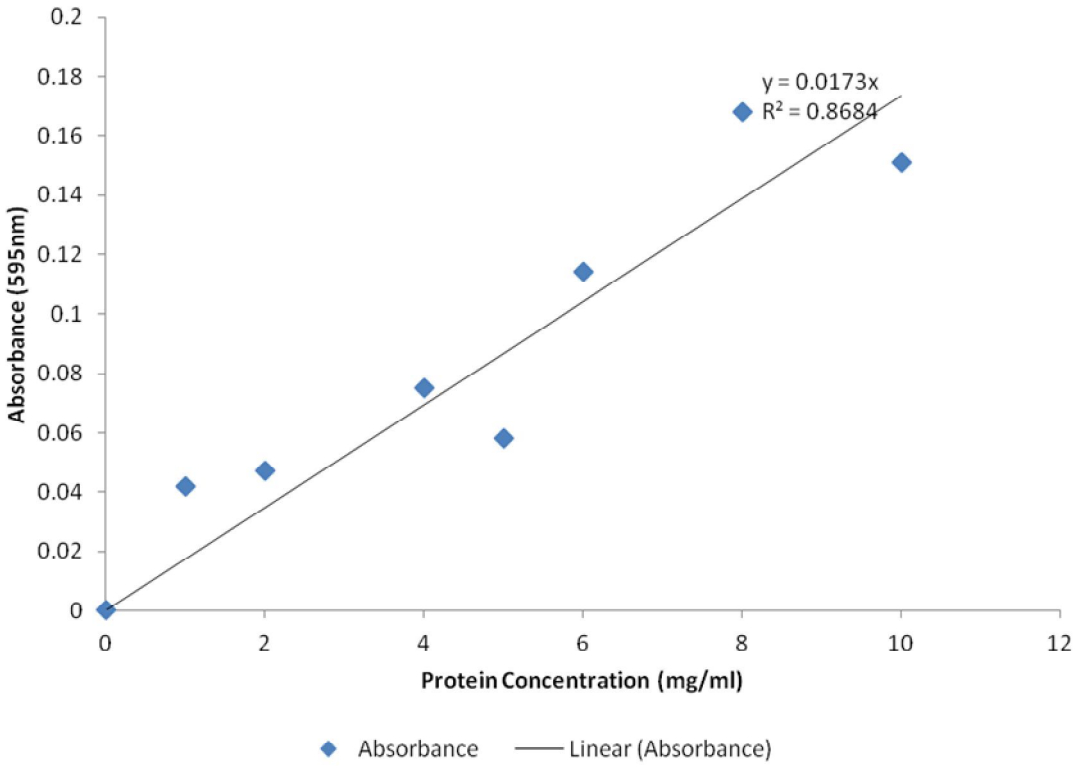
APPENDIX 3

Table showing the hemmaglutination titre , total protein and specific activity various fractions collected after ion exchange chromatography (II).

Fraction	Absorbance 280nm	Hemmaglutination titre	Salt gradient	Total protein	Specific activity
1	0.055	0	0.01	3.178	0
2	0.079	0	0.01	4.566	0
3	0.048	0	0.01	2.775	0
4	0.041	0	0.01	2.370	0
5	0.043	0	0.01	2.486	0
6	0.037	0	0.01	2.139	0
7	0.017	0	0.1	0.983	0
8	0.059	0	0.1	3.410	0
9	0.048	0	0.1	2.775	0
10	0.026	0	0.1	1.503	0
11	0.013	0	0.1	0.752	0
12	0.067	0	0.1	3.872	0
13	0.02	0	0.2	1.156	0
14	0.004	0	0.2	0.231	0.007
15	0.045	0	0.2	2.601	0
16	0.038	0	0.2	2.196	0
17	0.036	0	0.2	2.081	0
18	0.037	0	0.2	2.139	0
19	0.038	0	0.3	2.197	0
20	0.00915	0.08	0.3	5.289	0.267
21	0.019	0	0.3	1.098	0
22	0.016	0	0.3	0.924	0
23	0.018	0	0.3	1.041	0
24	0.021	0	0.3	1.214	0
25	0.032	0	0.3	1.850	0
26	0.023	0	0.3	1.330	0

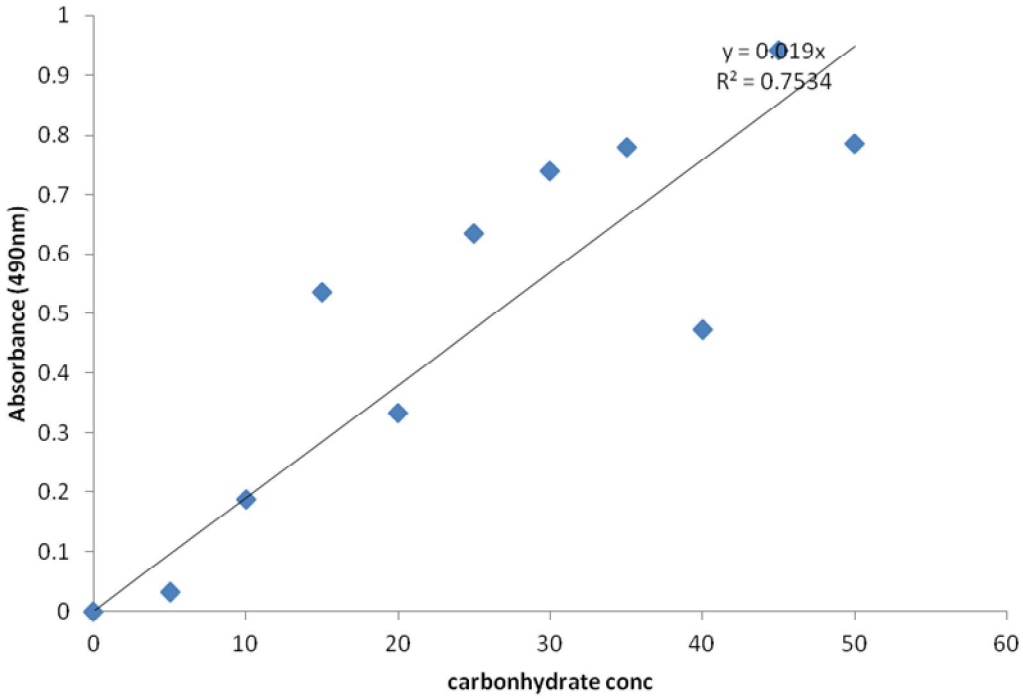
APPENDIX 4

Protein standard curve



APPENDIX 5

Carbohydrate standard curve



APPENDIX 6

Preparation of fixative solutions: The following two gel fixing solutions were prepared as outlined.

Reagent	**Gel fixing solution 1**		**Gel fixing solution 2**	
	Volume	**Final conc.**	**Volume**	**Final conc.**
Ethanol	50ml	50% (v/v)	60ml	30% (v/v)
Glacial acetic acid	10ml	10% (v/v)	-	
Water	40ml		140ml	
Total volume	100ml		200ml	

APPENDIX 7

Preparation of staining solutions: The following four gel staining solutions were prepared using the reagents provided in the kit.

Reagent	**Sensitizing solution**	**Staining solution**	**Developing solution**	**Stop solution**
Sensitizer concentrate	0.4ml	-	10µL	-
Staining reagent	-	4ml	-	-
Developing reagent	-	-	10ml	-
Stop reagent	-	-	-	8ml
Water	To 100ml	To 100ml	To 100ml	92ml
formaldehyde	-	54µL	27µL	-
Total volume	100ml	100ml	100ml	00ml

APPENDIX 8

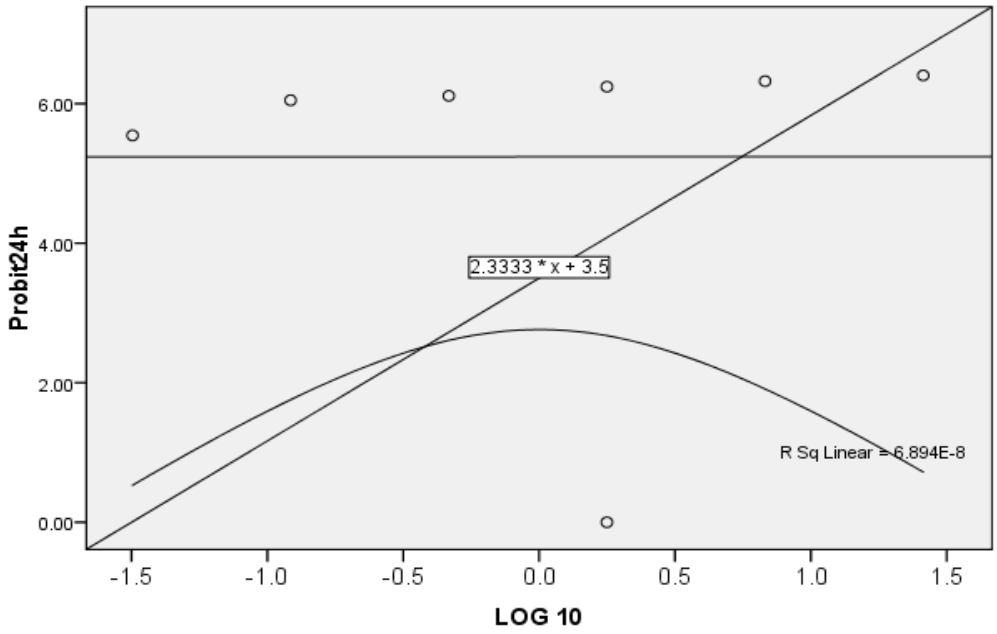
Aliquots of various strength solutions added to 100 ml water to yield final concentration

Initial solution %	PPM	Aliquot (ml)	Final concentration (PPM) in 100ml
1.0	10 000.0	1.0	100.0
		0.5	50.0
		0.1	10.0
0.1	1000.0	1.0	10.0
		0.5	50.0
		0.1	1.0
0.01	100.0	1.0	1.0
		0.5	0.5
		0.1	0.1
0.001	10.0	1.0	0.1
		0.5	0.05
		0.1	0.01
0.0001	1.0	1.0	0.01
		0.5	0.005
		0.1	0.001
0.00001	0.1	1.0	0.001
		0.5	0.0005

		0.1	0.0001

APPENDIX 9

Probit curve



APPENDIX 10

Pecentage mortality of *culex quinquifasciatus* larvae at different concentrations after 24 hours exposure

	Concentrations mg/L (After 24hrs)									
Sample	Control	0.0001	0.001	0.01	0.1	1	5	10	50	
Crude ex.	0	20	70.67	50.00	62.50	75.00	80.00	82.00	86.00	88.00
Gel I	0	2.67	16.00	16.00	28.00	42.67	50.00	58.67	64.00	68.00
Gel II	0	1.33	32.00	33.30	34.67	37.33	49.33	49.33	60.00	65.33
Ion I	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.67
Ion II	0	0.00	0.00	0.00	0.00	0.00	1.33	1.33	2.67	4.00

APPENDIX 11

Pecentage mortality of *culex quinquifasciatus* larvae at different concentrations after 48 hours exposure

	Concentrations mg/L (After 48hrs)									
Sample	Control	0.0001	0.001	0.01	0.1	1	5	10	50	100
Crude ex.	0	33.33	70.67	85.33	86.67	89.33	90.67	93.33	93.33	96.0
Gel I	0	6.67	24.00	41.33	42.67	54.67	56.00	62.67	68.00	74.67
Gel II	0	4.00	36.00	37.33	41.33	46.67	56.00	60.00	60.00	69.33
Ion I	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.33	10.67
Ion II	0	0.00	0.00	0.00	0.00	0.00	1.33	4.00	9.33	9.33