DEVELOPMENT OF CHROMATOGRAPHIC BIOSEPARATIONS BASED ON LECTINS AND SUPERMACROPOROUS AFFINITY CRYOGELS.

by

MOLOKO IVONNE RALETJENA

RESEARCH DISSERTATION

Submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

in

BIOCHEMISTRY

in the

FACULTY OF SCIENCE AND AGRICULTURE

(School of Molecular and Life Sciences)

at the

UNIVERSITY OF LIMPOPO

SUPERVISOR: PROFESSOR I NCUBE

CO- SUPERVISOR: PROFESSOR L J MAMPURU

2012

DECLARATION

I declare that the dissertation hereby submitted to the University of Limpopo, for the degree of **Master of Science** in **Biochemistry** has not been previously submitted by me for a degree at this or any other University; that it is my work in design and in execution, and that all material contained therein has been duly acknowledged.

_____ _____

Raletjena M I (Ms.) **Date**

Student Number: _____

DEDICATION

I would like to dedicate this work to: My late Grandmother, who taught me that even the largest task can be completed if it is done one step at a time.

ACKNOWLEDGEMENTS

I have worked with a great number of people whose contribution in assorted ways to the research deserves special mentioning.

1. In the first place I would like to record my gratitude to Prof I Ncube, who undertook to act as my supervisor despite his other academic and professional commitment. His passion for science, guidance and support from the initial to the final level has exceptionally inspired and enriched my growth as a student and a researcher.
2. I gratefully acknowledge Prof L J Mampuru, for his co-supervision. I am greatly indebted to his crucial contribution at all stages of this project through inspiration, encouragement and knowledge of the subject.
3. I gratefully thank Dr Vusi Mbazima. In the midst of all his PhD work, he has kindly granted his time to assist in the laboratory and for answering some of my unintelligent questions about cell culturing and Western blots. I am proud to record that I had an opportunity to work with an exceptionally experienced scientist like him.
4. In my daily work I have been blessed with a friendly and cheerful group of fellow student colleagues Manape, Gloria, Lebo, Kgomotso, Maphuti, Kholo and Rebone. Each helped make my time in the labs more fun and interesting whilst providing a stimulating environment to learn and grow.
5. Special thanks go to Busiswa Kekana, Dr P Masoko, and Dr P Mokgotho. This study would have not been successful without their knowledge and inputs.
6. I would also like to convey thanks to the staff members in the Department of Biochemistry, Microbiology and Biotechnology for their various forms of support
7. I was delighted to interact with Prof N Nyazema, Prof R Howard, and Dr K Lucas, who have always instilled in me a level of confidence and a drive to pursue my studies.
8. I owe my most sincere gratitude to Marumo “Sekobo’’ who as a good friend was always willing to help and advice during happier and difficult moments.

9. My most sincere thanks and appreciation are also due to my family and my extended family, which has always fully supported me, encouraged and believed in me in all my endeavors. My mother has so lovingly and unselfishlessly cared for Lattie and Tumi.
10. I greatly appreciate Ngoako, for his continued love, support and encouragements. He has been a good listener to all my complaints and frustrations.
11. I acknowledge the financial support by Swedish International Development Agency (SIDA), Vlaamse Interuniversitaire Raad (VLIR) and Department of Water Affairs (DWA).
12. I am also grateful to the Department of Biotechnology, Centre for Chemistry and Chemical Engineering, Lund University, for the generous supply of epoxy-polyacrylamide (pAAm) monolithic cryogels.
13. Furthermore I offer my regards to all those who have not been mentioned here personally and have contributed in any respect in making this educational process a success. Maybe I could not have made it without their support.

Above all, I thank God for giving me the strength and for taking me this far. The Almighty continued to bless me and made everything possible.

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

LIST OF ABBREVIATION

Act D	actinomycin D
AAm	acrylamide
AGE	allyl glycidyl ether
ATCC	American type culture collection
ATP	adenosine triphosphate
Bax	Bcl-2 associated X protein
BCA	bicinichonic acid
Bcl-2	B-cell lymphoma-2
BSA	bovine serum albumin
^{o}C	degrees centigrade (Celsius)
$CaCl_2$	calcium chloride
Cm	centimetres
CO_2	carbon dioxide
DMAEMA	2-(dimethylamino) ethyl methacrylate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELIFA	enzyme linked immunofiltration
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
g	grams
GLIA	glycoprotein-lectin immunosorbent assay
H	Hour
HCl	hydrochloric acid
HRP	horseradish peroxidase

IgG	immunoglobulin G
IMAC	immobilized metal affinity chromatography
kDa	kilo Daltons
$KHPO_4$	potassium hydrogen phosphate
LAC	lectin affinity chromatography
M	molar
mA	milliamperes
MBAm	N, N' methylene-bis-acrylamide
Mg	milligrams
ml	millilitres
mM	millimolar
$NaBH_4$	sodium borohydrate
NaCl	sodium chloride
Na_2CO_3	sodium carbonate
NaN_3	sodium azide
Nm	nanometres
NMR	nuclear magnetic resonance
NP-40	nonidet P-40
OPD	O-phenylenediamine
pAAm	epoxy polyacrylamide
PAL	*Pterocarpus angolensis* lectin
PBS	phosphate-buffered saline
PI	propidium iodide
PSL	*Pisum sativum* lectin
PSN	penicillin, streptomycin, neomycin
PVDF	polyvinylidene fluoride
RPMI	Roswell Park Memorial Institute

RCA-120	*Ricinus cummunis* agglutinin
RNase	ribonuclease
SBA	soybean agglutinin
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBS	tris-buffered saline
TEMED	N, N, N', N'–tetramethylenethylenediamine
µg	microgram
µl	microlitre
UV	ultraviolet
V	volts

ABSTRACT

Various cytomorphologic and biochemical markers of apoptosis are found in different compartments (plasma membrane, cytoplasm, nucleus, and mitochondria) of target cells. Although the plasma membrane is an easily accessible cellular compartment, relatively little is known about the changes in the expression of plasma membrane glycoproteins during apoptosis, and whether these changes could be used for detection of apoptosis. A critical element of this study was to purify lectins from crude homogenate on glycoprotein-cryogel affinity matrices, and later use the lectins to detect changes on the cell surface of apoptotic cells. *Pterocarpus angolensis* seed lectin was extracted and fractionated using ammonium sulphate precipitation. The 60 % ammonium sulphate pellet was dissolved in saline azide and purified using Sephadex G-75 affinity chromatography. A 28 kDa lectin was retarded within the column and appeared as a short and broad peak on the chromatogram. Traditionally, Sephadex G-75 column are used predominantly for size exclusion, in this study, the column was used in a non-traditional way for affinity chromatography, as the purified protein is able to bind sugar moieties existing in the structure of Sephadex G-75. A single-step purification of *P. angolensis* seed lectin was achieved by directly applying unclarified *P. angolensis* crude extract to the pAAm-cryogel using fetuin as the affinity ligand. *Pterocarpus angolensis* extract fractionated into 2 peaks, which revealed a highly concentrated band on SDS-PAGE. The results also revealed that an increased binding of the lectin to the fetuin-cryogel matrices was also dependent on the time of incubation. This study suggested very low capacities of the cryogels for the protein due to low coupling sites on the matrix. Taking into account that lectins serve as invaluable tools in diverse area of biomedical research, this study proposed using specific plant lectins to follow the expression of plasma membrane glycoproteins during programmed cell death. Treatment of HL-60 cells with lithium and actinomycin D confirmed a time- and dose-dependent inhibition of proliferation and a decrease in proliferation, which suggest cell death of the treated cells. The observed cell death was further investigated for cellular and biochemical hallmark features of apoptosis, which has shown preferential binding of annexin V-FITC to phosphatidylserine and low molecular DNA ladder. Several FITC labelled lectins were used to detect changes in cell surface glycosylation that accompany apoptosis. This study

has shown amongst several FITC-labelled lectins that *T. vulgaris* lectin could intensively stain the membrane area of apoptotic cells suggesting that the expression of N-acetylglucosamine was significantly increased during actinomycin D induced apoptosis of HL-60 cells. Binding was shown to be specific because it was blocked by the corresponding inhibitory sugar. Thus, the method described in this study could be suitable for the detection of very early stages of apoptosis by recognizing the cell surface carbohydrates of apoptosis

CHAPTER 1

INTRODUCTION

Lectins are carbohydrate-binding proteins of non-immune origin that are capable of specific recognition and reversible binding to specific sugar moieties of glycoconjugates without enzymatically modifying them. Consequently, lectins are widely employed in biochemical research as carbohydrate-specific reagents (Sengbusch, 2003; Hossain *et al*., 2004).

Lectins have attracted much interest primarily because they serve as invaluable tools in diverse area of biomedical research (Davidson and Stewart, 2004). Because of their unique carbohydrate binding properties, lectins are useful for the separation and characterization of glycoproteins, glycopeptides and glycolipids, and following the changes that occur in the cell surfaces during physiological and pathological processes from cell differentiation to cancer. Lectins are also useful in histochemical studies of cell and tissues, tracing neural pathways, typing blood cells and bacteria, fractionation of lymphocytes and bone marrow transplantation (Hossain *et al*., 2004; Gabor *et al*., 2004). These carbohydrate binding proteins are also useful tools as ligands in affinity chromatography (Bakalova and Ohba, 2002; Monzo *et al*., 2007). Affinity chromatography is the most selective type of chromatography employed in bioseparation techniques or studies.

Successful separation by affinity chromatography requires that biospecific ligand is available and that it can be covalently attached to a chromatographic bed material called a matrix (Arvidsson, 2002). It is important that the biospecific ligand (antibody, enzyme, lectin or receptor protein) retains its specific binding affinity for the substance of interest (antigen, substrate, glycoprotein or hormone). Thus lectins can be used as a ligand for purifying the respective binding substance, for example, polysaccharides, glycoproteins and cell receptors (Lucas, 2005).

At present, immobilized metal affinity chromatography (IMAC) is one of the most widely employed techniques for the purification of proteins from plant material (Nandakumar and Mattiasson, 1999; Putnam *et al*., 2003). This is by far the most preferred approach when the intended application is preparative scale (Putnam *et al*., 2003; Kumar *et al*., 2005), and when

the plant material raises special challenges including phenolics, tannins, and of course proteases (Nandakumar and Mattiasson, 1999). In these cases a quick and convenient first separation step is essential (Kumar *et al*., 2005).

Cell affinity chromatography has become an alternative for cell biologists and biotechnologists, due to a considerable need both in biomedical research and diagnostic medicine for specific separation of discrete population of cells from a mixture (Putnam *et al*., 2003; Kumar *et al*., 2004). Due to its low cost and simple operation cell affinity chromatography is by far the most effective separation system when the intended use is preparative scale (Kumar *et al*., 2003). However, cell affinity chromatography also entails great difficulties due to limitations by the matrices used.

Cryogels, which are polymeric gels formed in moderately frozen systems have recently been developed as new separation matrices for applications in various bioseparation processes (Arvidsson *et al*., 2003; Kumar *et al*., 2006). These continuous chromatographic columns have been produced by radical co-polymerization of acrylamide (AAm) with allyl glycidyl ether (AGE) or 2-(dimethylamino) ethyl methacrylate (DMAEMA) and cross-linker N, N' methylene-bis-acrylamide (MBAAm) in moderately frozen systems. The ice crystals formed after freezing perform as porogen (pore forming agent). The cryogels have a continuous system of interconnected macropores with a size of 10- 100 µm and show a very low flow resistance and allow unhindered diffusion of solutes of any size (Arvidsson *et al*., 2003).

Supermacroporous cryogels have been successfully used for fractionation of anti IgG coated human blood lymphocytes with protein-A as the ligand (Kumar *et al*., 2003; Kumar *et al*., 2006), chromatography of microbial cells using an ion exchange [2-(dimethylamino) ethyl] ligand (Arvidsson *et al*., 2002), and direct capture of $(His)_6$-tagged lactate dehydrogenase from crude homogenates (Arvidsson *et al*., 2003). In most of the applications using cryogel matrices, affinity was used as the primary choice because of the high capacity and specificity, stability of the ligand in dealing with the crude extract and simple ligand coupling chemistry. The use of cryogels as a tool for purification of plant lectins whereby glycoconjugates are ligands and fractionation of cell population with relatively cheap lectins as affinity ligands has potential in biomedical and biotechnology processes.

CHAPTER 2

LITERATURE REVIEW

2.1 LECTINS

2.1.1 Sources of plant lectins

Lectins can be found in plants (e.g., Fabaceae), animals (e.g., snails), and microorganisms (Vornholt *et al*., 2007). Although lectins are widely distributed in nature, plant lectins are the most abundant. In plants, lectins can be detected predominantly in seeds or in storage organs, whereas the content of different plant species differs in a wide range (Rudiger and Gabius, 2001). Plant lectins are found mainly in the seeds of protein bodies and may constitute up to 10% of the total seed protein. The lectins are also found in the vegetative parts such as the stem, bark, roots and leaves and more than one lectin may be present in the same tissue. Plant lectins have received enormous interest and, hence, they have been well defined with regard to their carbohydrate specificities (Hirabayashi, 1997).

2.1.2 Biophysical and biochemical properties of plant lectins

Plant lectins are divided into five groups according to the monosaccharide for which they exhibit the highest affinity. These are: group 1: D - mannose / D – glucose group; group 2: D – galactose / N-acetyl – D – galactosamine group; group 3: N – acetyl –D – glucosamine group; group 4: L - fucose group and group 5: N – acetylneuraminic acid group (Goldstein *et al*., 1997).

Amongst the plant lectins, legume lectins are the largest and best characterized groups with more than 70 lectins reported towards the end of the millenium (Hirabayashi, 1997). Comparisons of both the amino acid sequences and the 3-dimensional structures (physico-chemical properties) of the legume lectins show a very high degree of homology, yet they exhibit very distinct differences in their sugar specificities (Sharon and Lis, 1990). For this reason, this large family of homologous proteins is highly suitable for use as a model system for studying the principles behind protein-carbohydrate recognition (Loris *et al*., 2003).

Legume lectins typically consist of dimers or tetramers of subunits of relative molecular mass of 25 to 30 kDa that are held together by non-covalent interactions (Srinivas *et al*., 2001). Each subunit has one sugar-binding site and binds calcium and manganese ions which are required for the sugar-binding activity. The sugar-binding site consists of four loops designated A, B, C and D. Loop D provides additional interactions, and is possibly the determinant of the monosaccharides specificities in legume lectins (Barre *et al*., 2001; Loris *et al*., 2002).

2.1.3 Biomedical and biotechnological application of plant lectins

In recent years, lectins have attracted much interest primarily because they serve as invaluable tools in diverse area of biomedical research (Davidson and Stewart, 2004). In the case of plant lectins there are two basic ideas: on one hand, they could play a role in the protection against animal, fungal or bacterial predators. On the other hand, these lectins possibly interact with storage proteins or enzymes of the plant. It is also debated that the lectins may be involved in symbiotic procedures between plants and bacteria (Rudiger and Gabius, 2001).

2.1.3.1 Applications of lectins in histochemistry

Lectin histochemistry is an integral part of diagnostic histopathology and glycosciences (Aoki *et al*., 1993). Despite the emerging new molecular techniques, which grant an insight into the genetic codes and their messages, glycohistochemistry using lectins is one of the most important techniques in biomedical sciences (Gabius *et al*., 1993). Lectins have been intensively used in histochemical techniques for cell surface characterization and as histochemical markers (Amadeo *et al*., 2005; Hedemann *et al*., 2007), since they can indicate differences in cell surfaces due to changes in glycosylation, a process that is post-translated and therefore cannot be detected using molecular techniques (Aoki *et al*., 1993).

Lectins are capable of binding sugar residues of cell walls or membranes. This reaction changes the physiology of the cell wall and influences the metabolism of the living cell (Sharon and Lis, 1993). Some lectins of plants stimulate immune system by unspecific activation of the T-cells (e.g., the lectins of *Canavalia ensiformis*, Con A binds to different membrane receptors and leads to proliferation of these cells) while others causes specific

agglutination of specific cell types (e.g., erythrocyte groups) and therefore are valuable tools for blood typing (Khan *et al*., 2002).

2.1.3.2 Lectin glycoconjugate interaction

Virtually all vertebrate cell surfaces are endowed with a glycocalyx, a carbohydrate coating composed of membrane glycoproteins, glycolipids, glycosaminoglycans, together referred to as glycans or glycoconjugates. The carbohydrate residue of glycoconjugates may encode a large repertoire of information per monomer unit, differing in the number and type of sugar residues, sequence of sugar moieties, type of anomeric linkage, presence or absence of branches, and the type and amount of sialic acid (Bilyy and Stoika, 2003).

Because of their structural complexity and variability, cell surface carbohydrates serve as recognition signals (Sharon and Lis, 1990). The structural modification of oligosaccharides domain of cell-surface glycoconjugates occurs during normal cell development and is correlated with a host of physiologically important functions, such as cell recognition, behavior, growth, contact inhibition and differentiation. The structural diversity encodes unique signals that are recognized by divalent or polyvalent lectins on the surface of opposing cells in a complementary way analogous to ligand receptor interaction. In addition, soluble lectins and glycoproteins may act as bridges by binding to carbohydrates on opposing cells and extracellular matrix (Mody *et al*., 1995).

A number of studies have shown that cell surface carbohydrates are modified upon malignant transformation, tumor cell differentiation, and metastasis (Dabelsteen *et al*., 1991; Lu and Chaney, 1993; Ravindranath *et al*., 2006). The level of expression of cell-surface carbohydrate-binding proteins (endogenous lectins) is also altered during cancer and metastasis (Mody *et al*., 1995).

2.1.3.3 Applications of lectins in cell separation

Differential binding of some lectins to the surface of mammalian cells suggests that during the different stages of development, expression of surface glycoconjugates changes, and that expression of receptors on the surface of cells for lectins could be used to identify different

subpopulation of cells (Porras *et al*., 2005). Thus, lectins represent excellent tools for isolation of cellular populations. For example: Peanut agglutinin (*Arachis hypogaea*) recognizes immature human lymphocytes (Reisner and Gan, 1983); T and B splenocytes are fractioned with lectins from *Glycine max* (soybean agglutinin, SBA) (Reisner and Gan, 1983; Porras *et al*., 2005) and *Helix pomatia* is employed for identification and isolation of human T cells (Reisner and Gan, 1983).

By virtue of their binding specificities, lectins have been used as reliable biochemical, cytochemical and histochemical probes in the study of subtle differences in the cell surface glycoconjugates on malignant and non-malignant cells that are otherwise nondetectable with available monoclonal antibodies. Since most lectin reacts specifically with terminal, non-reducing sugars of glycoproteins and glycolipids component of the cell membrane, they can be used to characterize these surface glycoconjugates on the basis of the monosaccharides that inhibit the binding. Some lectins exhibit pronounced anomeric specificity, e.g. Concavalin A (Con A) lectin binds specifically to α-form of sugars (Goldstein and Porets., 1986).

2.1.3.4 Lectin affinity chromatography

Lectin affinity column chromatography is a method of choice for the fractionation and purification of oligosaccharides, especially N-linked oligosaccharides (Iams, 1999). Using lectin affinity, it is easy to separate structural isomers and to isolate oligosaccharides based on specific features. Further, serial lectin column chromatography, when various lectin columns are used at the same time, can afford a very sensitive method for fractionation and characterization of extremely small amounts of oligosaccharides (Lewandrowski *et al*., 2005).

Lectin affinity chromatography offers a tool that aids purification of cell surface glycoconjugates in sufficient quantities so that studies addressing their structural elucidation could be carried out. It has several advantages over the conventional biochemical methods such as immunoprecipitation and/ or immunoaffinity chromatography, used for the purification of various glycoconjugates (Satish and Surolia, 2001). Apparently, the affinity of lectins to bind complex carbohydrates is often an order of magnitude higher than that for monosaccharides. In addition, the haptenic sugars used to inhibit lectins may not reflect the structural nature of the glycans recognized with high affinity, as the binding of two competing ligands can be deduced

from the law of mass action and depends on two parameters: The concentration of the ligands and their affinity to the lectins (Monzo *et al*., 2007).

Lectin affinity chromatography (LAC) is an "all or nothing" interaction as in all other biospecific interactions (i.e., affinity interactions) (Iams, 1999). In other words, a glycoconjugate that has no affinity towards a given lectin will pass through the column unretained, thus eluting within the void volume of the column. Conversely, a glycoconjugate that exhibits affinity towards the immobilized lectin will be strongly retained by the lectin affinity ligand and will only elute from the column by a step gradient with the eluting mobile phase containing a haptenic sugar (i.e., inhibitor) thus exiting the column at exactly the dead time of the column (Amano *et al*., 2001).

In principle, lectin affinity chromatography is similar to other types of chromatography. A mixture of glycoconjugates is chromatographed on a matrix with a particular lectin immobilized on it. The glycoprotein with a specific sugar sequence get adsorbed onto the matrix because of its interaction with the immobilized lectin, while other glycoproteins are washed off by the buffer and collected as the breakthrough. Washing is continued until no more proteins comes out of the column by using the buffer alone, as monitored by the absorbance at a particular wavelength, against a suitable blank. The adsorbed glycoprotein is eluted from the column using a specific sugar with a complimentary structure (Satish and Surolia, 2001).

2.1.3.5 Lectins as affinity ligands

Lectins can be immobilized on a solid surface matrix (such as agarose) for lectin affinity electrophoresis or for lectin affinity chromatography to generate 'fingerprints' of oligosaccharides on the basis of their elution characteristics, or to isolate and further analyze their structures by means of other biochemical methods.

Lectins may also be immobilized on solid surface for glycoprotein-lectin immunosorbent assay (GLIA) by direct, indirect, or sandwich application (Kottgen *et al*., 1993) or can be immobilized on nitrocellulose or nylon membranes for enzyme linked immunofiltration (ELIFA). Lectins can be derivatized with radioactive isotopes, fluorochromes, biotin, reporter

enzymes and group specific reagents (Mody *et al*., 1995) to render them detectable during anatomical, histochemical, and cytochemical studies. The oligosaccharide-lectin interaction may also be studied using NMR spectroscopy.

2.1.4 *Pterocarpus angolensis* lectin (PAL)

2.1.4.1 Nature of *P. angolensis* seed lectin

The fruit of *Pterocarpus angolensis* bloodwood tree is a pod that is covered with spiny bristles and surrounded by an orbicular, slightly lobed wing. The entire fruit is between 50-150 mm in diameter, and carried on a stalk about 10 cm, in length. Pods generally contain a single seed although this may change with location (Graz, 2004).

The seed from the tropical legume *Pterocarpus angolensis* contains a lectin, the *Pterocarpus angolensis* lectin (PAL) that belongs to the mannose/glucose (Man/Glc) specific group, which contains several well-studied members such as Concanavalin A (Loris *et al*., 2002; Loris *et al*., 2003). The carbohydrate-binding site contains a classic Man/Glc type specificity loop, whereas the metal binding loop on the other hand is of the long types, different from what is observed in other Man/Glc specific legume lectins (Loris, 2002).

The known *Pterocarpus* lectins are glucose/mannose–specific dimeric proteins (28 kDa subunits molecular mass) (Loris *et al*., 2003). The lectin is stable over a wide range of pH values, and very resistant to denaturation (Beeckmans *et al*., 2002).

2.1.4.2 Binding of *P. angolensis* seed lectin to fetal erythrocytes

In a study of haemagglutinins of trees and shrubs of Zimbabwe, it was shown that the lectin present in the seeds of *P. angolensis* was haemagglutinating only protease- treated human cord erythrocytes and not adult erythrocytes (Beeckmans *et al*., 2002), from which it was concluded that *P. angolensis* seed lectins react preferentially with antigens related to the i-blood group, which is expressed in fetal systems and is found on cord erythrocytes. The lectin also binds glycoproteins such as horseradish peroxidase and ovalbumin (Ndamba *et al*., 1994). D-glucose,

D-mannose, and N-acetyl-D-glucosamine inhibit binding of the lectin from seeds of the mukwa (*P. angolensis*) tree to fetal erythrocytes (Loris *et al*., 2003).

2.2 CELL AFFINITY CHROMATOGRAPHY

Nanoparticle and cell separation is a new challenge within the field of bioseparation. Among the important minor cell populations include stem cells in haematological samples, fetal cells in maternal blood, and residual leukaemic or tumour cells from patients in clinical and morphological remission or antigen-specific lymphocytes (Kumar *et al*., 2005).

Isolation of these cell subsets in sufficient numbers with high purity and viability is commonly required both in clinical practice and in basic research as for further genomic and proteomic studies (Kumar *et al*., 2004). Thus developing an effective separation system for large-scale cell separation has been a challenging research goal for cell biologists and biotechnologists (Kumar *et al*., 2003).

At present, the most widely employed technique for the isolation of pure living cell populations from biological fluids and tissues are affinity-based separations which make use of monoclonal antibodies that bind to cell-specific surface glycoproteins. Among the affinity separation techniques, affinity chromatography based on immunoabsorption of target cells to macroscopic surface derivatized with monoclonal antibodies or other specific ligands, has several advantages (Braun *et al*., 2000). These include high specificity for the target cells; separation of cells with high yields in short periods of time, and simple operation (Braun *et al*., 2000; Putnam *et al*., 2003). The limitation of this is strongly influenced by the load, surface density, and orientation of the immobilized antibody (Kumar *et al*., 2005).

Due to its low cost and simple operation, cell affinity chromatography is by far the most preferred approach when the intended application is preparative scale separation (Kumar *et al*., 2003; Kumar *et al*., 2004). Cell affinity chromatography relies on the interactions of cell-surface bound molecules and their complementary ligands (monoclonal antibodies or lectins). If a ligand is covalently bound to a chromatographic support and is accessible to the passing cells, the cells possessing the appropriate cell surface molecules will bind to the chromatographic support, and will be easily removed from the solution. However, cell

chromatography is different from traditional protein chromatography and entails great difficulties (Kumar *et al*., 2003).

As separation subjects, cells are relatively large and are rather fragile and sensitive to shear stress. Moreover, because of the multipoint attachment of the cells to the ligand, on the adsorption matrix, their recovery in viable form posses some problems. Thus, for cell affinity chromatography the main requirement is the design of a suitable matrix, which can be used successfully for the cell separation by addressing the above challenges (Kumar *et al*., 2003).

2.2.1 Supermacroporous cryogel matrices

Recently, continuous polymeric chromatographic columns with pore size large enough to accommodate cell debris and even whole cells without being blocked have been developed (Arvidsson *et al*., 2002; Arvidsson *et al*., 2003). Cryogels, which are polymeric gels formed in moderately frozen systems have been developed recently as new separation matrices for applications in various bioseparation processes (Arvidsson *et al*., 2003; Kumar *et al*., 2006).

2.2.1.1 Preparation of supermacroporous matrices

The supermacroporous monolithic cryogels have been produced by radical co-polymerization of acrylamide (AAm) with allyl glycidyl ether (AGE) or 2-(dimethylamino) ethyl methacrylate (DMAEMA) and cross-linker N, N′-methylene-bis-acrylamide (MBAAm) in moderately frozen systems (Plieva *et al*., 2004). The polymerization goes on in a small non-frozen (the so-called non-frozen liquid micro-phase) medium where the dissolved substances (monomers and initiator) are connected. The gel formation in the liquid microphase and crystals of the frozen solvent after partial freezing perform as porogen (pore forming agent) .Thus shape and size of the ice crystals determine shape and size of the pores (Figure 1), which depends on how fast the system is frozen, provided that other parameters (e.g., concentration of the dissolved substances, volume and geometrical shape of the sample) remain the same (Arvidsson *et al*, 2002).

The dissolved monomers and initiators are concentrated in a small fraction of a non-frozen fluid in which polymerization proceeds efficiently despite that the whole system looks like a

frozen ice block. After melting, a continuous gel is formed. The gels have a continuous system of interconnected macropores of a 10-100 μm with sponge-like morphology (Plieva *et al*., 2004). The cryogels show a very low flow resistance and allow unhindered diffusion of solutes of any size (Savina *et al*., 2005). The pore size depends on the initial concentration of reagents in the solution and the freezing conditions (Hanora *et al*., 2006).

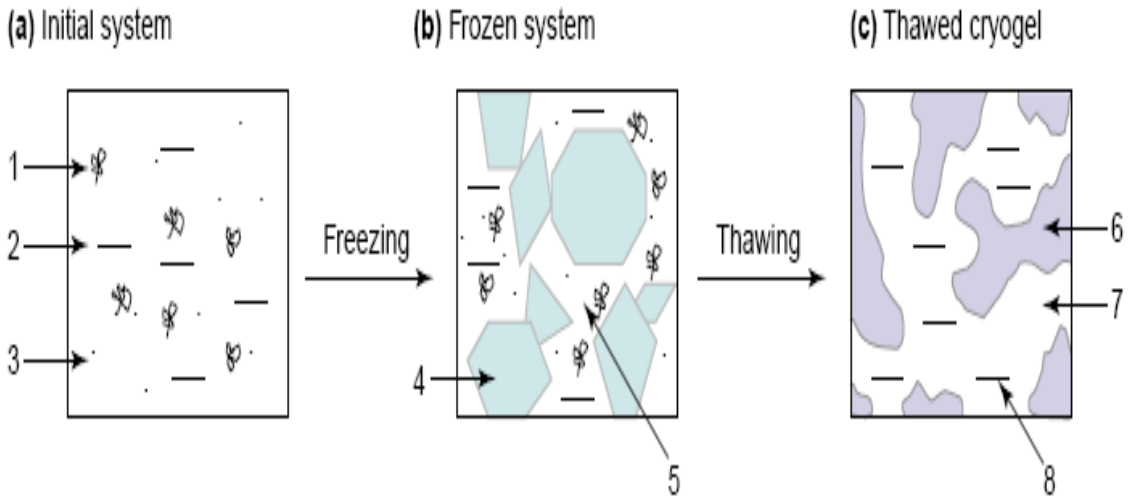


Figure 1: Preparation of supermacroporous cryogel matrices. 1, macromolecules in a solution; 2, solvent; 3, low-molecular solutes; 4, polycrystals of frozen solvent; 5, unfrozen liquid microphase; 6, polymeric framework of a cryogel; 7, macropores; 8, solvent. (Lozinsky *et al*., 2003).

2.2.1.2 Advantages of supermacroporous matrices over convectional methods

Traditional packed-bed chromatography with immobile stationery phase, despite its elegance and high resolving power, has a major limitation: incapability of processing particulate-containing fluids, for example human blood. Blood cells are trapped between the beads of the chromatographic carrier resulting in increased flow resistance of the column and complete blockage of the flow. Expanded-bed chromatographic set-up overcomes the problem of handling particulate-containing fluids (Babac *et al*., 2006). However the high shear stresses could be detrimental for the integrity of blood cells.

It is attractive to have a packed-bed chromatographic carrier with pores large enough to accommodate large particle e.g. blood cells without being blocked. The high porosity of the cryogels makes them appropriate candidates as the basis for such supermacroporous chromatographic materials (Lozinsky *et al*., 2003). Polymeric gels have also shown applications in many different areas of biotechnology including use as chromatographic materials, carriers for the immobilization of molecules and cells, matrices for electrophoresis and immunodiffusion, and as gel basis for solid cultural media (Lozinsky *et al*., 2003).

The demands for a chromatographic matrix to be suitable for nanoparticle separation are (i) macroporosity, (ii) high chemical and physical stability, (iii) possibilities of stable coupling of biospecific ligands and (iv) the capability to withstand rough elution and regeneration conditions. The degree of macroporosity needed of course depends on the size of the target particle. The pores should be at least 5-10 times larger than the particles to be separated (Arvidsson, 2002).

Continuous bed (monolithic) chromatographic columns represent a new class of chromatographic materials introduced as an alternative to traditional packed bed columns (Savina *et al*., 2005). The structure of interconnected monolith, and low mass transfer limitations endures cryogel packed columns with interesting chromatographic properties (Plieva *et al*., 2004). The pore size plays a crucial role in chromatographic performance of monolithic columns, thus allows unhindered mass transport of solutes of practically any size (Dainiak *et al*., 2004). Transport of solutes inside the monolithic cryogel column mainly proceeds due to convection rather than diffusion. Columns have very low backpressure with more than 90% of the column volume being composed of interconnected supermacropores (Plieva *et al*., 2004).

Supermacroporous matrices produced by the cryotropic gelation technique provide an attractive tool for manipulating cells in a chromatographic mode (Plieva *et al*., 2004). With a proper choice of ligand capable of specific interactions with the surface of the cells, it is possible to bind cells quantitatively to the supermacroporous matrix and elute with recoveries as high as 70-80% without impairing viability of the cells (Arvidsson *et al*., 2002).

One advantage of having a continuous matrix compared to a beaded one is the absence of void volume between the beads, and the lowest theoretical void volume of ideally packed uniformly sized beads. Macroporous matrices can be used as ion exchange resins, support for combinatorial synthesis, solid support reagents and chromatographic support media. (Arvidsson, 2002). These matrices can also be used to directly capture enzymes of interest from crude homogenates, and mostly as matrices in cell affinity chromatography (Arvidsson *et al*., 2002; Arvidsson *et al*., 2003). On the other hand, monolithic columns or matrices have an advantage over conventional beaded matrices operating at high flow rates as high as 100 cm/h with the mass transfer governed by convention inside the pores of the monolithic column (Hanora *et al*., 2006).

2.2.1.3 Use of cryogels in bioseparation techniques

It is attractive to have a packed-bed chromatographic carrier with pores large enough to accommodate cell debris and even whole cells without being blocked. As such, large (on molecular size) objects as cells and cell debris have a negligible diffusivity, the transport of them inside the column can only be convective. Hence, the desired bed should have a continuous system of macropores rather than to be composed of porous beads because in the later case the convective flow is mainly restricted to the inter-particle volume inside the column (Arvidsson *et al*., 2003).

The large pore size in cryogel in combination with highly interconnected morphology allows substances of different molecular weights, bovine serum albumin (69 kDa), blue Dextran (2000 kDa), or even microbial cells (3-10 μm) to pass through the cryogel matrix without retention (Savina *et al*., 2005). The cryogels were used for fractionation of human blood lymphocytes with protein-A as the ligand (Kumar *et al*., 2003; Kumar *et al*., 2006). Similarly the effect of ligand coupling and matrix architecture on cryogels were also investigated and demonstrated with binding of CD 19^+ B-lymphocytes and $CD34^+$ KG-1 human tumor cells (Kumar *et al*., 2005) on the cryogel affinity matrices. The binding and separation strategy was based on the interactions between the Protein A coupled to the gel matrix and the IgG molecules which were used for labelling the cells through specific surface receptors (Ahlqvist *et al*., 2006).

2.3 CANCER AND APOPOPTOSIS

Cancer is a universal problem and a leading cause of death in both male and female in different parts of the world (Yi *et al*., 2003; Reddy *et al*., 2003). The disease is still a clinical problem and has a significant social and economic impact on the human health care system (Cheng *et al*., 2005). Cancer can occur at any age, but it is more common as people grow older. Not all cancers, however, are confined to advancing age, as the most tragic cancers are found in children. Although there are over one hundred different kinds of cancer, only a few occur frequently. The most frequent is skin cancer, followed by cancers of the lung, colon, breast, and prostate. Several agents cause cancer (chemicals and radiation) and are called carcinogens (Reddy *et al*., 2003).

2.3.1 Carcinogenesis

Cancerous cells results due to a single cell that begins to proliferate abnormally due to disorders that occurs in the normal processes of cell division controlled by the genetic material (DNA) of the cell. Human cancers may result from exposure to environmental carcinogens which include the natural and man-made chemicals, radiation, chromosomal rearrangement, tumour suppressor genes and viruses (Reddy *et al*., 2003).The abovementioned factors are triggered by:

(i) Incorrect diet, which is estimated at least 35 % of all cancers worldwide (Hussain *et al*., 2003; Reddy *et al*., 2003)

(ii) Genetic predisposition, which leads approximately 20 % of cancer cases (Yi *et al*., 2003).

(iii) Environmental factors, which is associated with majority of cancers (Reddy *et al*., 2003).

The stages of carcinogenesis include initiation, promotion and progression. The first stage involves a reaction between the cancer-producing substances (carcinogen) and the DNA of the tissue cells; there may be a genetic susceptibility. The second stage occurs very slowly over a period ranging from several months to years. During this stage, a change in diet and lifestyle can have a beneficial effect for a person not to develop cancer during their lifetime. The last

stage involves progression and spread of the cancer, at which point diet may have less impact (Reddy *et al*., 2003).

2.3.2 Cell Death

The control of cancer may benefit and restore the lives of many individuals; therefore, there is a need to utilize different scientific approaches to come up with solutions that can assist in the prevention and possibly a cure for this deadly disease. Currently, the search and development for drugs which are safe and limits resistance is the major priority in cancer therapy. An ideal suitable chemotherapeutic agent or drug should induce cancer cell death without showing any cytotoxic effects on the normal cells or triggering any inflammatory reactions (Yi *et al*., 2003)

Extensive research in the past few years has revealed that cell death, whether at the single cell level, the tissue level or organism level, is as important to life as survival. In effect, the cell death is a process of replacement, renewal, replenishment, regeneration and revitalization of tissue (Zimmermann *et al*., 2001), thus an integral part of normal development and maturation cycle. Cell death is said to occur in two alternative modes depending on the stimuli. Apoptosis, a controlled type of cell death that can be induced by a variety of physiological stimuli and pharmacological agents, and necrosis, as unordered and accidental form of cell death that occurs in response to a number of independent biochemical events that are activated by severe cell injury and depletion of cell energy (Lawen, 2003; Cheng *et al*., 2005).

2.3.2.1 Apoptosis

Apoptosis, also referred to as programmed cell death mode of cell death, plays a crucial role in embryonic development, metamorphosis, hormone-dependent atrophy and tumour growth as a physiological event, regulating cell number and eliminating damaged cells (Min-Liang *et al*., 1996). It is a normal physiological process in which a cell actively terminates itself by the destruction of vital cellular components or DNA via various molecular signaling pathways (Wyllie, 1987). Apoptotic cell death is characterized by organized enzymatic slices of the intercellular content including DNA and proteins before the actual cell death. Cell death is also characterized by specific morphological patterns including plasma membrane bebbling, nuclear condensation, chromatin condensation, endonucleolytic degradation of DNA (Thompson,

1995; Cummings and Schnellmann 2002; Jin *et al*., 2006), and cell shrinkage and fragmentation into membrane coated vesicles called apoptotic bodies due to condensation of the cytoplasm (Wyllie, 1987; Hengartner, 2000). This membrane-bound apoptotic bodies are then engulfed by phagocytes of the immune system for clearance.

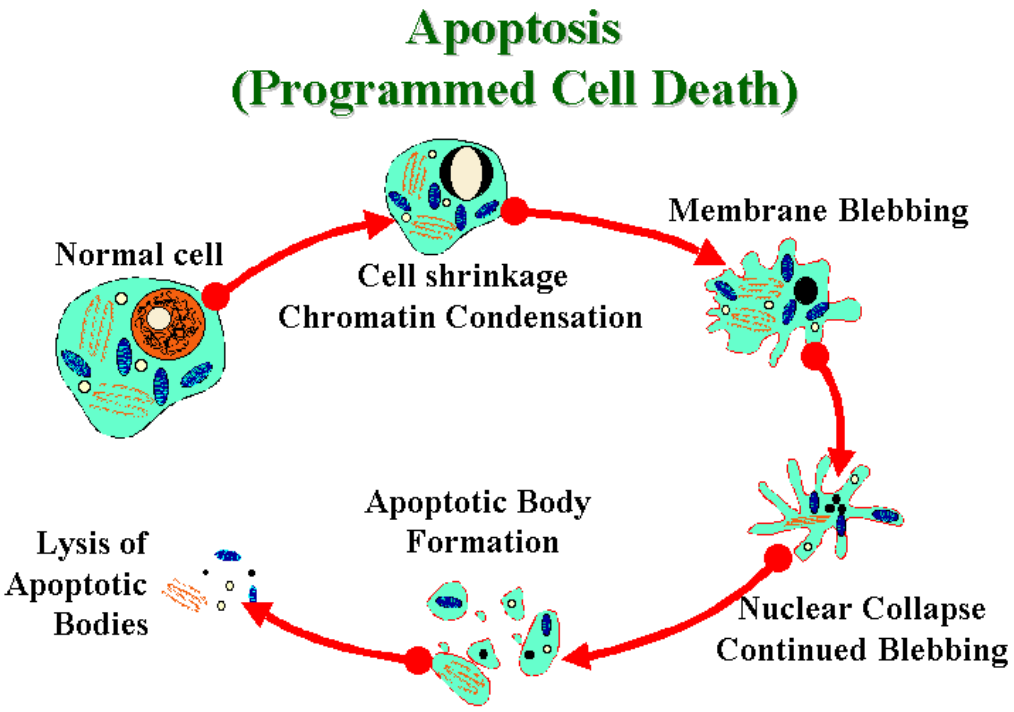


Figure 2: Morphological features of an intact cell undergoing apoptosis (Kamakoff , 2007).

Programmed cell death is accompanied by the expression of different biological markers in the cytoplasm (activation of caspases and the appearance of cytochrome C) (Fujimura *et al*., 2002; Chang and Yang, 2000), expression and/ or translocation of pro- and anti apoptotic proteins of Bcl-2 family members of the mitochondria (Reed, 1994; Cho and Choi; 2002), DNA fragmentation in the nucleus (Lawen, 2003), and plasma membrane(externalization of phosphotidyl serine)(Vermes *et al*., 1995; Van Engeland *et al*., 1996; Lawen, 2003).

The plasma membrane of the apoptotic cells is believed to remain relatively intact. The most well documented changes in the plasma membrane are the translocation of phosphotidyl serine (normally confined to the inner leaflet) to the external side of the plasma membrane during the early phases of apoptosis, which can be detected by FITC-annexin V specific binding (Appelt

et al., 2005; Zhang *et al*., 2000) and the expression of Fas and tumour necrosis factor membrane receptors in the apoptotic cells (Bilyy and Stoika, 2003). Annexin-V is a Ca^{++}-binding protein able to interact with negatively charged phospholipids, such as phosphotidyl serine, in a Ca^{++} dependent manner (Bilyy and Stoika, 2003).

However there are several reports showing that there are further alterations during the late phases of apoptotic cell death, leading to the recognition by additional adaptor molecules. Bilyy and Stoika (2003) and other investigators (Heyder *et al*., 2003) demonstrated an increased expression of α-D-mannose and β-D-Galactose rich plasma membrane glycoprotein in apoptotic cells. It was then suggested that such glycoproteins can be novel plasma membrane markers of apoptotic cells. Their expressions were demonstrated in cell lines of various tissue origins, and associated with various inducers of apoptosis. It is also believed that glycoproteins can also be used for isolation of apoptotic cells from a mixture of cell population (Bilyy *et al*., 2004).

It was further proposed (Billy and Stoika, 2003) that specific plant lectins can follow the expression of plasma membrane glycoproteins at apoptosis, found that the expression of *Pisum sativum* lectin (PSL, α-D-Mannose specific) and β-D-Galactose specific *Ricinus cummunis* agglutinin (RCA-120); were significantly increased in plasma membrane of apoptotic murine leukemia cells of L1210 line. Thus, an elevated expression of plasma membrane glycoproteins may be used for detection of apoptotic cells and for the quantitative estimation of their number in a cell population (Bilyy and Stoika, 2003).

2.4 RATIONALE

A critical element of modern process biotechnology, biomedical research and diagnostic medicine is the separation and purification of bioparticles such as discreet population of healthy and pathological cells, viruses and proteins. Understanding cell developmental pathways also becomes increasingly significant.

Prior studies have demonstrated that a wide range of antitumour agents induce apoptosis in human tumour cell *in vitro*. Apoptosis is a physiological or programmed cell death that removes elderly and impaired cells so they can be replaced with the fresh cells.

Chromatography of apoptotic cells based on the specific glycoproteins in the plasma membrane that undergo redistribution during apoptosis has been identified as a suitable process for the enrichment of apoptotic cells.

Lectins can be used as markers to bind specifically to carbohydrate component of the glycoprotein molecule during programmed cell death, thus improving the separation of apoptotic from non-apoptotic cells. Since cells have very low diffusion coefficients due to the large size and could be forced inside the pores only by convective flow, the ideal separation technique should provide a good yield with high purity in a short time while maintaining function and minimizing manufacturing costs. Thus, developing an effective separation system for large scale separation and improvement of apoptotic cells has been a challenging research goal for cell biologists or biotechnologists.

2.5 AIMS AND OBJECTIVES

Aim

- The main aim of this study was to develop a chromatographic bioseparations of lectins and cells based on glycoprotein and lectin supermacroporous matrices.

Objectives

- To evaluate the glycoprotein supermacroporous matrices on fractionation of *P. angolensis* seed lectin from crude homogenates.

- To investigate binding of different lectins on apoptotic HL- 60 cell lines.

CHAPTER 3

MATERIALS AND METHODS

3.1 Equipment

The following equipment was used during the study. CO2 Incubator (NAPCO model, Instrulab cc, Johannesburg, S.A), Centrifuges (model GS-15R, Allegra X 22R Beckmann Instruments Inc., Fullerton, USA), Light and Fluorescence Microscope (Zeiss, Germany), Power-pack (Bio-Rad Laboratories), Spectronic Genesys 5 spectrophotometer (Milton Roy Company, USA), Beckman Coulter DTX 880 multimode detector and Syngene image analyzer (Vacutec, RSA).

Econo UV monitor, Econo pump, fraction collector model 2110 and ChemiDoc XRS image analyzer were from Bio-Rad Laboratories Inc., USA.

3.2 Supermacroporous cryogel matrices, seeds, cells, culture media and biochemicals

Mature seeds from *Pterocarpus angolensis* were obtained from forestry commission of Zimbabwe, epoxy-polyacrylamide (pAAm) monolithic cryogels were given as a gift from the Department of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, Sweden and HL-60 cell cultures were purchased from American Type Culture Collection, Rockville, USA.

Sodium azide, lithium chloride, actinomycin D, sodium chloride, ammonium sulfate, ammonium persulfate, bovine serum albumin (BSA), Sephadex G-75, bromophenol blue, acrylamide, Coomassie-Brilliant Blue R-250, sodium dodecyl sulfate (SDS), glycine, sodium di-hydrogen phosphate, di-Sodium hydrogen phosphate, dimethyl sulfoxide, mannose, mucin type II (purified from porcine stomach), mucin type III (crude from porcine stomach), fetuin (from fetal calf serum), thyroglobulin (from porcine), Trizma base, N,N,N',N'-tetramethyl-ethylendiamine (TEMED), 2-mercaptoethanol, BCA protein assay reagent kit, N-acetyl glucosamine, propidium iodide, O- phenelynediamine tablets, Triton X-100, sodium acetate,

Tween-20, annexin V-FITC, anti-rabbit IgG peroxidase, hydrogen peroxide, phenol, hydroxyquinoline, trypan blue, RNase, proteinase K and sodium borohydrate were bought from Sigma Chemical Company (St. Louis, MO, USA). Acetic acid, glutaraldehyde, chloroform, isopropanol, and hexane were bought from Merck. RPMI-1640 media, Foetal bovine serum (FBS) were bought from Highveld Biologicals (Pty) Ltd, Lyndhurst, South Africa.

Penicillin, streptomycin and neomycin, (PSN) mixture was from Gibco, Auckland, New Zealand. Sodium chloride, ethanol, methanol, KHPO4, sodium hydroxide, sodium carbonate, acrylamide, bisacrylamide, Nonident P-40, sodium orthovanadate, agarose, fat-free powdered milk, ethidium bromide and sodium bicarbonate were purchased from Saarchem (PTY) LTD. (Saarchem (Pty) Ltd, Midrand, South Africa). Glycerol was from NT Laboratory supplies (Pty) Ltd, JHB, South Africa and EDTA was from BHD, Gauteng, South Africa.

Goat anti-mouse Bcl-2 antibody, goat anti-mouse Bax antibody, goat anti-mouse IgG-HRP conjugated secondary antibodies and western blotting luminol reagent (Santa-Cruz Biotechnology Inc., Santa-Cruz, CA, USA). Protease inhibitor cocktail tablets were from Roche Diagnostics GmbH, Mannheim, Germany and PVDF membrane was from Pall Corporation, Pensacola, FL, USA.

3.3 Experimental methodology

3.3.1 Extraction and ammonium sulphate fractionation of *P. angolensis* seed lectin

Mature seed of *P. angolensis* were decoated and ground to a meal using a blender. Oil in 50 g seed meal was removed by extracting with 250 ml of hexane (5 ml hexane/g of seed meal) for 1 h while stirring at room temperature. The seed meal was dried on filter paper at room temperature until all the hexane had evaporated. Protein was extracted using saline azide (0.9 % NaCl and 0.02 % sodium azide in distilled water) (5 ml of solution/g of fat free seed meal) while stirring overnight at 4 °C. The extracted protein was kept at -20 °C until required for use. The protein extract was thawed, filtered through two layers of cheesecloth to remove any floating fatty particles and later centrifuged for 30 min at 3080 x *g*.

The supernatant was subjected to 30 % ammonium sulphate precipitation by adding ammonium sulphate (164 g ammonium sulfate/1000 ml solution) to the saline extract, with stirring at 4°C for 1 h. The precipitated protein was then centrifuged at 3080 x *g* for 30 min. The supernatant was further subjected to 60 % ammonium sulfate fractionation by adding ammonium sulphate (181 g ammonium sulfate/ 1000 ml solution) while stirring at 4 °C for 1 h. The solution was centrifuged at 3080 x *g* for 30 min. The pellet containing lectin was dissolved in minimum volume of saline azide to obtain a crude extract which was stored at 4 °C.

3.3.2 Purification of *P. angolensis* seed lectin using Sephadex G-75 affinity chromatography

The Sephadex G-75 column was prepared by swelling Sephadex G-75 in an excess of saline azide (10 g/100 ml saline azide) and incubating overnight at 4 °C. A Sephadex G-75 column (1.5 cm x 35 cm) was equilibrated with saline azide solution at a flow rate of 0.4 ml/min using Bio-Rad Econo pump. The dissolved pellet (4 ml) was loaded onto the column and absorbance of the fractions monitored at 280 nm. Fractions were collected every 10 min. Based on the previous observations in this affinity adsorption, the lectin fractions peak were from the retarded broad peak at the end of the chromatogram.

3.3.2.1 SDS- PAGE according to Laemmli (1970)

The purity of the lectin fractions was determined on a 15 % SDS-PAGE, according to Laemmli, (1970). The protein samples (30 µl) were mixed with SDS-PAGE sample buffer (Laemmli, 1970), boiled for 15 min and 20 µl carefully loaded into the wells of the gel. Two of the wells were loaded with 15 µl and 10 µl of molecular weight markers and crude extract respectively. The electrophoresis was run initially at 100 volts, and then increased to a constant voltage of 200 volts when samples were out of the stacking gel. After the run was completed, the gel was removed from the plates and stained with Coomasie Brilliant Blue stain (0.1 % Coomasie Brilliant Blue R-250 in 40 % methanol and 10 % acetic acid) with gentle shaking at room temperature. The stain was poured off and the gel was destained in water by heating in a microwave for about 5 min.

3.3.3 Preparation of lectin and fetuin cryogel affinity column

The epoxy-polyacrylamide (pAAm) supermacroporous monolithic cryogel was prepared according to method used by Kumar *et al*. (2003). A 2 ml pAAm monolithic cryogel matrix was placed in a glass column (1 cm x 4.5 cm), washed with water until the pH was close to neutral, and then washed with 20 ml of 0.2 M Na_2CO_3 (20 ml). A solution of ethylenediamine (0.5 M in 2 M Na_2CO_3) was recycled through the column at a flow rate of 1 ml/min. The column was washed with water until the pH was close to neutral and then washed with 20 ml of 0.1 M sodium phosphate buffer, pH 7.2. A solution of glutaraldehyde (30 ml) (5 % in 0.1 M sodium phosphate buffer pH 7.2) was applied to the column at a flow rate of 1 ml/min in recycle mode for 5 h. The derivatized supermacroporous matrix with functional aldehyde groups was used for coupling *P*. *angolensis* lectin. The lectin (260 µg/ml, 12 ml) or fetuin (2 mg/ml) in 0.1 M sodium phosphate buffer pH 7.2 was recycled through the column at a flow rate of 1 ml/min at 4 °C for 24 h. Finally, 30µl of freshly prepared $NaBH_4$ solution (0.1M in sodium phosphate buffer pH 9.2, 30 ml) was passed through the column at a flow rate of 1 ml/min for 3 h in a recycled mode. The $NaBH_4$ solution to reduced Schiff's bases formed between the protein and the aldehyde-containing matrix.

3.3.4 Evaluation of lectin- cryogel using fetuin/ ovalbumin

The *P. angolensis* lectin-cryogel affinity column (1 cm x 4.5 cm) prepared in section 3.3.3 was used for evaluate the cryogels using fetal calf serum fetuin and ovalbumin. The column was first washed thoroughly by passing 0.1 M phosphate buffer; pH 7.2 at a flow rate of 0.7 ml/min. Fetuin or ovalbumin (0.4 ml) at 1 mg/ml was loaded onto the column at a flow rate of 0.7 ml/min in separate experiments and the elution monitored by measuring the absorbance of the fractions at 280 nm using Beckman Coulter DTX 880 multimode detector. After the glycoprotein solution had entered the column, the glycoproteins were incubated within the cryogel matrix for 15 min at room temperature before elution. The bound glycoproteins were eluted first with 0.3 M mannose in phosphate buffer solution, followed by elution with 0.1 M acetic acid in phosphate buffer, with fractions being collected after every minute.

3.3.5 Purification of *P. angolensis* seed lectin on fetuin-Cryogel

The fetuin-cryogel column (1.5 cm x 32 cm) prepared as described in section 3.3.3 was equilibrated with saline azide solution at a flow rate of 0.4 ml/min with absorbance monitored at 280 nm. The crude extract (0.05; 0.1; 0.5 and 2 ml), which was previously prepared (as described in section 3.3.1) in saline azide was loaded onto the column. The unbound protein was eluted with saline azide solution, followed by elution of the bound lectin with 0.3 M mannose in saline azide solution. Fractions were collected after every three minutes.

3.3.6 Modifications on the fetuin cryogel matrix for the purification of crude homogenate of *P. angolensis* seed lectin

The capacity of fetuin-cryogel matrices prepared as described in section 3.3.3 was increased by few modifications to the method used by Kumar *et al*. (2003). Theoretically, further improvement of the binding efficiency could be expected using a longer extension or spacer arm (Dainiak *et al*., 2005). The 2 ml cryogel column was connected to a pump and washed with water at a flow rate of 1 ml/min followed by 0.2 M Na_2CO_3 (20 ml). A solution of ethylenediamine (0.5 M in 2 M Na_2CO_3) was recycled through the column at a flow rate of 1 ml/min. The column was washed with water until the pH was close to neutral, and further washed with 20 ml 0.1 M sodium phosphate buffer, pH 7.2. A solution of glutaraldehyde (30

ml; 5 % in 0.1 M sodium phosphate buffer, pH 7.2) was applied to the column at a flow rate of 1 ml/min in recycle mode for 5 h. Fetuin (2 mg/ml; 20 ml) in 0.1 M sodium phosphate buffer, pH 7.2, was recycled through the column at a flow rate of 1 ml/min at 4 °C for 24 h. The coupling procedure was repeated twice by recycling 30 ml of glutaraldehyde (5 % v/v in 0.1 M sodium phosphate buffer, pH 7.2) through the column at a flow rate of 1 ml/min for 5 h, followed by the solution of fetuin (2 mg/ml; 20 ml) in 0.1 M sodium phosphate buffer, pH 7.2, at a flow rate of 1 ml/min at 4 °C for 24 h. Finally, the freshly prepared $NaBH_4$ solution (0.1 M in sodium carbonate buffer, pH 9.2; 30 ml) was applied to the column at a flow rate of 1 ml/min for 3 h in recycle mode. The fetuin cryogel matrix was then used to purify 2 ml of the crude homogenate. The purity of the lectin fractions was determined on SDS-PAGE as described in section 3.3.2.1

3.3.6.1 ELISA assays for specific elution profiles of PAL

The lectin fractions collected in section 3.3.6 were coated on a 96-well flat-bottom microtiter plate (50 µl of lectin fraction and 50 µl of carbonate–bicarbonate buffer, pH 7.6) and incubated overnight at 4 ºC. The plate was blocked with BSA (1 % in PBS and 0.1 % Tween 20, followed by washing three times with PBT (PBS and 0.1 % Tween 20) on a shaker at room temperature. This was followed by addition of anti-PAL antiserum in PBS-BSA (0.1 % BSA in PBS) at a dilution of 1:1000, and further incubation for 1 h on shaker at room temperature. The plate was washed 3 times with PBT. The anti-rabbit IgG-peroxidase (1: 1000) in PBS-BSA was added and incubated for 1 h on shaker. The plate was further washed with PBT. An O-phenylenediamine (OPD) substrate was freshly prepared by dissolving an OPD tablet (0.4 mg/ml) in 20 ml 0.1 M phosphate buffer with 2 µl of hydrogen peroxide added just before adding the OPD solution to the plate. The substrate (200 µl) was added to the plate and the absorbance at 405 nm read immediately using the Beckman Coulter DTX 880 multimode detector.

3.3.7 Cell culture and cell viability analysis of HL-60 cell lines

The human myeloid leukemia cell lines, HL-60 cells were cultured in 90 % RPMI-1640, supplemented with 10 % fetal bovine serum (FBS) and 1 % PSN (penicillin G, streptomycin and neomycin) antibiotics. The cells were seeded at 2 x 10^5 cells/ml in a 50 ml flask, and grown at 37 °C under a humidified, 5 % CO_2 atmosphere. Cell growth and viability was monitored using trypan blue dye exclusion assay (Weisenthal *et al*., 1983). The cells in logarithmic growth (8-10 x 10^5 cells/ml) phase were used for further experiments.

3.3.8 Treatment of cells to induce apoptosis

Cells in logarithmic growth-phase were seeded at 2 × 10^5 cells/ml, and dispensed within a 250 ml culture flask in 80 ml volumes. The cultured cells were treated by incubating with different concentrations of either lithium chloride (0, 10 and 20 mM) or actinomycin D (0, 1 and 5 µg/ml) for 0, 12, 24, 48 h and 0, 6 and 24 h respectively. During the experimental intervals 1 ml was withdrawn from the flasks and analyzed for cell viability and cell population/ density. The other 20 ml from the culture flasks were transferred into centrifuge tubes placed on ice and centrifuged at 100 x *g* for 5 min. The cell pellet was washed 3 times with ice-cold PBS, pH 7.4 and stored the pellet at -20 °C until use.

3.3.9 Assays for apoptosis

3.3.9.1 Cell count and viability analysis

To investigate the growth inhibition effects of different concentrations of lithium chloride and actinomycin D on HL-60 cells, the cells were withdrawn from experimental flasks and subjected to trypan blue dye exclusion assay. A 1 ml of the withdrawn cells was stained with 2 drops of trypan blue dye and then placed on a haemocytometer. Cells that excluded the dye were taken as viable and those that did not exclude the dye and stained blue were taken as non-viable when counted using the haemocytometer under the light microscope.

3.3.9.2 Qualitative analysis of DNA fragmentation by agarose gel electrophoresis

Apoptosis of HL-60 cells was confirmed by detection of fragmentation of chromosomal DNA using the classic DNA ladder method. The HL-60 (2 x 10^5 cells/ml), treated as described in section 3.3.9 were harvested and washed using ice-cold PBS, pH 7.4. The cells were lysed by resuspending the cell pellet in 0.5 ml digestion buffer (5 mM Tris-HCl, pH 7.6; 1 mM EDTA; and 0.5 % Triton X-100). The lysate was clarified by centrifuging for 10 min at 14 000 x *g*. The pellet which contained high molecular weight DNA and cell debris was treated similarly but in different tubes from the supernatant that contained fragmented DNA. The supernatant was incubated with 0.5 mg/ml RNase at 37 ºC for 1 h, after which Proteinase K was added to a final concentration of 0.5 mg/ml. The tubes were further incubated at 50 °C with a gentle inversion of the tubes in order to mix periodically. The cell lysates were then cooled at room temperature. Extraction of DNA from the cell lysates was done using 500 µl of phenol–chloroform mixture (1: 1) and centrifuged twice at 8940 x *g* for 5 min at 4 °C, followed by precipitation with 1/10^{th} volume of 3 M sodium acetate (pH 5.2) and equal volume of ice-cold isopropanol at -20 ºC for 24 h. The DNA pellet was collected by centrifugation at 17500 x *g* for 5 min, washed with 70 % ethanol, dried at room temperature and then resuspended in 50 µl of sterile distilled water. The purity of the DNA was determined by spectrophotometric analysis at 260 and 280 nm. Approximately 5 µl (1 part) of DNA was mixed with 5 parts of gel loading buffer (10 mM EDTA, pH 8.0; 0.25 % Bromophenol blue; and 50 % glycerol). Samples were heated at 70 °C and loaded onto a 1.5 % agarose gel containing 0.5 µg/ml ethidium bromide. The gel was electrophoresed at 2 volts/cm for about 3 h after which it was photographed under UV light.

3.3.9.3 FITC-annexin V staining of HL-60 cell line

The early stage of apoptosis was confirmed by staining with FITC-annexin V conjugate. Apoptosis was induced at a 5 x 10^5 cells/ml by addition of different concentrations of lithium chloride and actinomycin D (as described in section 3.3.8) for 6 to 24 h and 1 to 4 h respectively. The cells at 2 x 10^5 cells/ml were harvested and gently washed with cold PBS. The cell pellet was then resuspended in 100 µl of a freshly prepared annexin V incubation reagent containing 990 µl of binding buffer (0.1 M HEPES, pH 7.4; 1.4 M NaCl and 25 mM $CaCl_2$) and 10 µl of 1 mg/ml annexin V-FITC in a buffer containing 10 mM Tris, 0.1 % NAN_3

and 1 % BSA . The apoptotic cell suspension was either transferred into a plastic V-shaped microtiter plate or eppendorf tubes depending on the method of analysis. The eppendorf tubes and the plate were incubated in the dark for 10 min. In a separate cell suspension, a 0.1 ml propidium iodide (PI) solution in binding buffer was added. All the reaction tubes were washed twice with PBS and then observed under the microscope or measured fluorescence using a microtiter plate reader using an excitation wavelength of 495 nm.

3.3.9.4 Western blot analysis

HL-60 cell lines were treated with 5 µg/ml of actinomycin D at different time intervals (0- 6 h). Cells were harvested and lysed in lysis buffer(2 mM Tris-HCl, pH 8.0; 1 % Nonidet P-40; 13.7 mM NaCl; 10 % glycerol; 1 mM sodium orthovanadate and protease inhibitor cocktail tablets) for 20 min on ice and later centrifuged using a microfuge at 17500 x *g* for 15 min at 4 ºC. Aliquots of the supernatants were used to quantify the protein concentrations using BCA assay (Smith *et al*., 1985) and the remainder was stored until required for use. Equal amounts of protein from the aliquots (15-30 µg/ml) were resolved on a 12 % SDS-PAGE. The proteins on the gels were then electro-blotted onto PVDF membrane using a blotting buffer (20 % methanol, 25 mM Tris and 192 mM glycine, pH 8.3) at 200 mA at 4 ºC for 2 h. Following electro- blotting, the membranes were blocked with TBS-T ween (0.05 % Tween 20 in 20 mM Tris-HCl and 200 mM NaCl, pH 7.4) containing 5 % fat-free powdered milk for 1 h at room temperature. The membranes were washed three times for 10 min each wash with wash buffer (TBS-Tween without milk), and then incubated overnight with specific primary goat anti-mouse Bcl-2 antibody (1: 1000) and goat anti-mouse Bax antibody (1:1000) in 0.05 % TBS-Tween at 4 ºC. The membranes were washed 3 times with wash buffer for 10 min each and further incubated for 1 h with goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1: 10000 in the blocking buffer. The membranes were washed again 3 times with the wash buffer for 10 min. The immunoreactive proteins were then detected using a Western blotting luminol reagent (Santa-Cruz Biotechnology) following the manufacturer's protocol. The protein bands were then viewed using a BioRad Chemi Doc XRS Image Analyzer.

3.3.9.5 Data presentation and statistical analysis

All values are expressed as mean ± standard deviation (SD). The data was analysed by one-way ANOVA followed by Dunnett's comparison test using GraphPad InStat Software. Differences between the means of control and lithium- and actinomycin D-treated cells were considered significant at $p \leq 0.05$.

3.3.10 FITC-Lectin binding studies

3.3.10.1 Screening of several lectins as biochemical markers of apoptosis

Several FITC-labeled lectins were used to determine which lectins bind the cell surface of apoptotic HL- 60 cells. Apoptosis was induced as described in section 3.3.8 using cells at a density of 5 x 10^5 cell/ml. Aliquots of 100 µl cell suspension at 3 x 10^5 cells/ml was added to a V-shaped microtitre plate and spun down using Beckman Allegra X 22R centrifuge at 190 x *g*. The FITC labeled lectins (*Pterocarpus angolensis*, Concanavalin A, Red kidney soybean, *Pisum savitum*, *Tritum vulgaris*, and *Helix pomatia*) at 1 mg/ml, or 0.25 % trypsin or PBS were added to both the treated and untreated cell suspension and incubated in the dark for an hour at room temperature. The cells were then washed thrice using ice-cold PBS. A volume of 100 µl was transferred into a flat bottom microtiter plate and fluorescence measured using a Beckman Coulter DTX 880 multimode detector. Another part of the cells were transferred onto slides and viewed under fluorescence microscope.

3.3.10.2 Determination of the titre for *T. vulgaris*

Apoptosis on the HL-60 cell lines was induced by incubation of cells (5 x 10^5) with 5 µg/ml of actinomycin D in a 50 ml cell culture flask. Following the incubation, the cells were pelleted and washed twice with PBS. A 100 µl of the cell suspension at 3 x 10^5 was added to eppendorf tubes and later stained with different concentrations (100 µg/ml-1 mg/ml) of FITC-*T. vulgaris* lectin in the binding buffer. The cells were then washed thrice using PBS, transferred onto slides and viewed under fluorescent microscope. The fluorescent intensity on the actinomycin D treated cells was used to determine the titre of *T. vulgaris* lectin.

3.3.10.3 Lectin sugar-inhibition tests

A volume of 100 µl of HL-60 cells (3×10^5) harvested after inducing apoptosis with 5 µg/ml of actinomycin D were incubated with several reaction mixtures for 30 min. The first reaction mixture contained 100 µl of PBS with 100 µl of 1 mg/ml FITC-*T. vulgaris* lectin; the second reaction mixture contained 100 µl of 2 mg/ml BSA with 100 µl of FITC-*T. vulgaris* lectin and the third reaction mixture contained 100 µl of 0.2 M N acetyl-glucosamine with 100 µl of FITC-*T. vulgaris* lectin. All the reaction mixtures were incubated with the appropriate buffer and sugar for 30 min prior to staining the cells. Following the incubation, the reaction mixtures were washed with appropriate diluents (reaction 1 and 2 used PBS, pH 7.4 whilst reaction 3 used 0.2 M N acetyl-glucosamine in PBS). The washing was repeated three times prior to observation under fluorescent microscope.

CHAPTER 4

RESULTS

4.1 Purification of *P. angolensis* seed lectin using Sephadex G-75 affinity chromatography

Purification of the lectin from the seed extract was achieved by the combination of ammonium sulphate fractionation and affinity adsorption chromatography on a Sephadex G-75 column. This was based on previous results that showed that the lectin could be eluted as a retarded peak on Sephadex G-75 column (Ncube, 2005; personal communication). Figure 3 shows that components of the redissolved 60 % ammonium sulphate pellet was separated into three major peaks. The first peak eluted as a high shouldered peak. The second peak eluted close but separate from the first peak, whereas the lectin peak was retarded within the column and appeared as a short and broad peak.

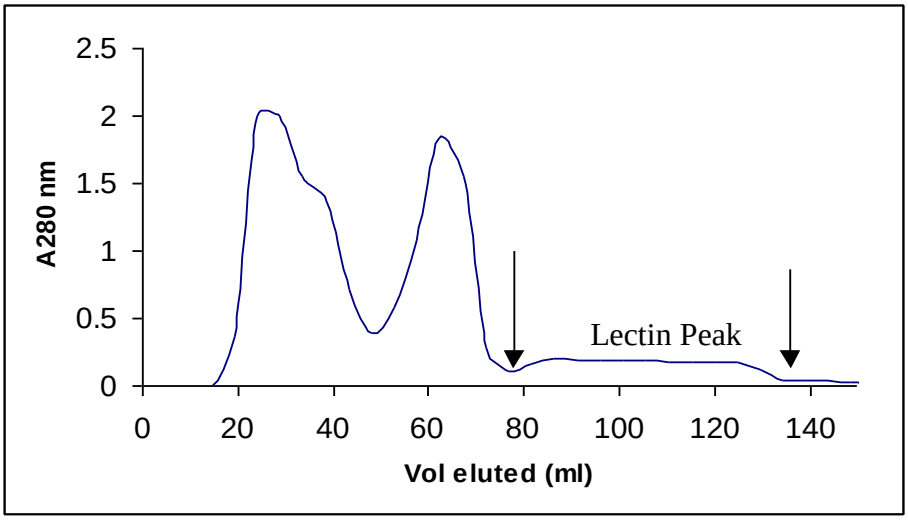


Figure 3: Elution profile of *P. angolensis* seed lectin on Sephadex G-75 affinity chromatography. The redissolved 60 % pellet was loaded onto Sephadex G-75 column (1.5 cm x 32 cm) and eluted at a flow rate 0.4 ml/min. Protein elution was monitored at 280 nm using BIORAD UV monitor. Arrows indicate lectin fractions.

After purification of the lectin from the crude extract, there was need to analyze the purity and homogeneity of the selected fractions. The lectin fractions collected from the retarded peak (fractions 45 to 51) that appeared at the end of the chromatogram were analyzed on a 15 % SDS-PAGE gel. It was evident that all the fractions collected contained a pure protein as noticed by single bands of approximately 28 kDa (Figure 4).

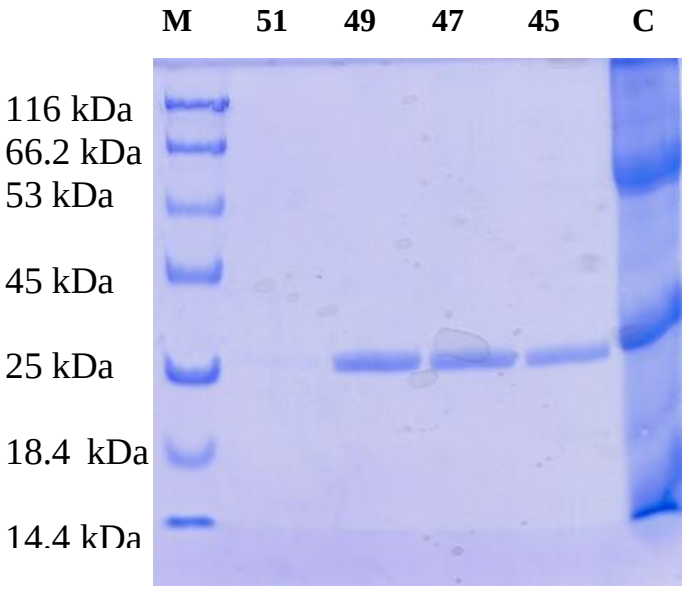


Figure 4: SDS-PAGE of purified lectin fractions collected from Sephadex G-75 column. Lane M: Molecular weight marker, Lanes 45, 47, 49, and 51: Retarded peak fractions obtained from Sephadex G-75 column. Lane C: Crude seed extract

4.2 Preparation and evaluation of lectin -cryogel affinity column

A sponge-like, macroporous cryogel matrix was produced by radical co-polymerization of different monomers in moderately frozen systems. The *P. angolensis* seed lectin was coupled to the pAAm supermacroporous cryogel matrix via a spacer arm through derivatization of the matrices with glutaraldehyde. To assay the activity of this lectin-supermacroporous column, glycoproteins fetuin/ovalbumin was used. Elution of the unbound glycoprotein was observed as the first distinct peak followed by elution of the bound protein, which resulted as a shorter peak (Figure 5). A further elution of the bound protein with acetic acid did not result in further elution of the bound protein.

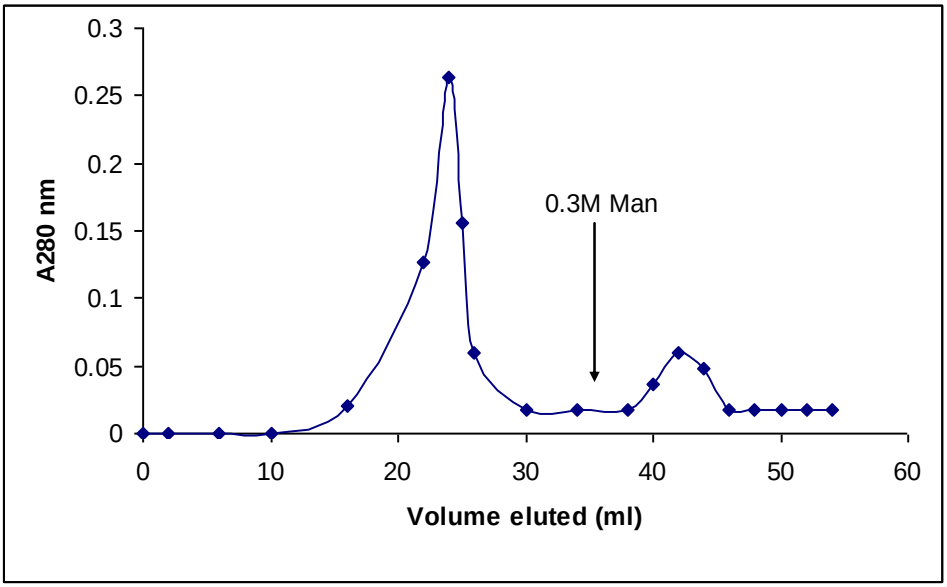


Figure 5: Elution profiles of 1 mg/ml glycoprotein on *P. angolensis* lectin supermacroporous affinity chromatography. Ovalbumin (0.4 ml) was loaded onto lectin-supermacroporous column (1 cm x 3.5 cm) and eluted at a flow rate of 0.7 ml/min. Protein elution was monitored at A_{280} nm. Arrow indicates the specific buffer used to elute the glycoprotein

4.3 Preparation and evaluation of glycoprotein-cryogel affinity column on purification for PAL from crude homogenates.

Different volumes of an unclarified *P. angolensis* crude extract were directly applied to the fetuin pAAm-cryogel affinity column without centrifugation or ammonium sulphate precipitation. The crude extract passed easily through the pores of 10-100 µm of the fetuin-cryogel matrices. The chromatographs shows that most of the impurities were removed with the wash buffer followed by desorption of the bound lectin by competitive elution using 0.3 M mannose in phosphate buffer pH 7.2 to displace the *P. angolensis* seed lectin from the fetuin cryogel matrix (Figure 6). The purity and homogeneity of the lectin fractions were assayed on a 15 % SDS-PAGE which revealed clear bands of approximate same size though with other small amount of contaminants (Figure 7).

Elution of different volumes of the unclarified crude extract with mannose recovered about 62 % of the protein with a purification factor of 5.2 (Table 1) when a 0.5 ml of the crude extract was loaded followed by a 39 % recovery when a 1 ml of the crude extract was loaded. A280 nm readings of the bound pool was independent of the volume loaded as compared with the unbound pool which showed a significant increase in the absorbance with an increase in the volume loaded onto the fetuin-cryogel matrix (Table 2).

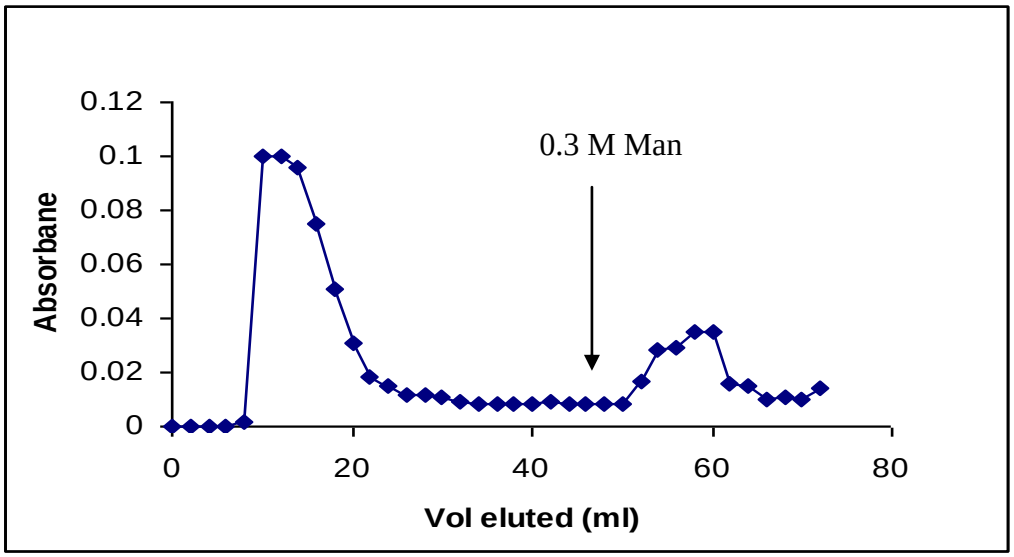


Figure 6: Elution profile of *P. angolensis* seed lectin from fetuin pAAm- supermacroporous column. Unclarified *P. angolensis* crude extract volume of 2 ml was loaded on fetuin cryogels (1 cm x 3.5 cm). Protein elution was monitored by measuring absorbance at 280 nm. The arrow indicates the specific buffer used to elute the lectin.

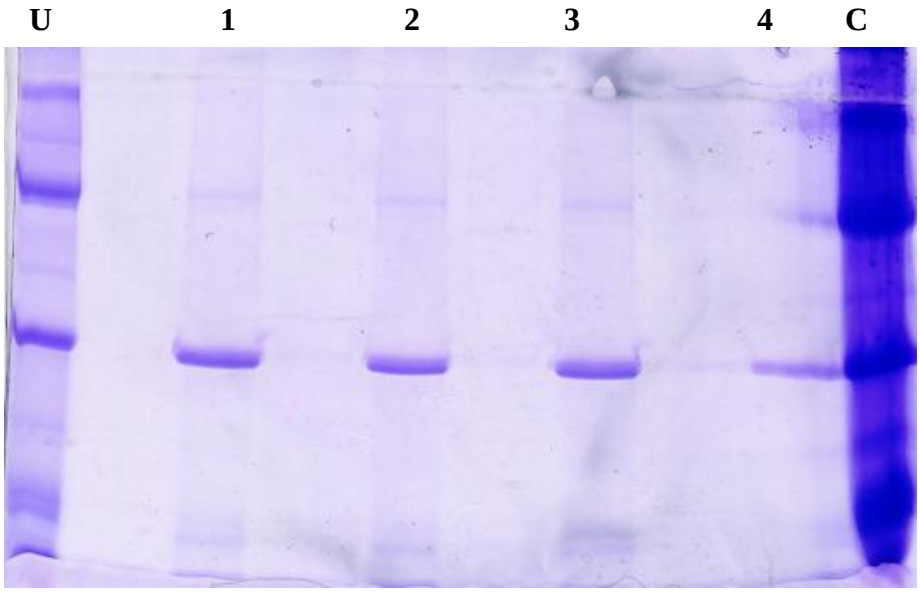


Fig 7: SDS-PAGE of purified lectin. Lane U: unbound protein, Lanes 1, 2, 3 and 4: Pool of bound fractions with a 2, 1, 0.5 and 0.05 ml respective loaded volumes. Lane C: Crude protein.

Table 1: Purification table of an unclarified *P. angolensis* seed lectin on fetuin-cryogel matrices

Sample	Vol (ml)	Activity U/ml	[protein] (mg/ml)	TA (U/ml)	Sp.Act u/mg	Yield %	Purification Fold
Crude	1	3.3×10^5	142	3.2×10^5	2.3×10^3	100	1
Crude	0.05	3.3×10^5	142	1.6×10^4	2.3×10^3	100	1
(0.05ml)	12	160	0.3	1.9×10^3	500	11.7	0.21
Crude	0.1	3.3×10^5	142	3.2×10^4	2.3×10^3	100	1
(0.1ml)	18	640	0.3	1.1×10^4	1.8×10^3	35.2	0.8
Crude	0.5	3.3×10^5	142	1.6×10^5	2.3×10^3	100	1
(0.5 ml)	20	5×10^3	0.4	1.0×10^5	1.1×10^4	62.5	5.2
Crude	2	3.3×10^5	142	6.5×10^5	2.3×10^3	100	1
(1ml)	25	1.0×10^4	0.4	2.5×10^4	2.4×10^4	39	10.6

Table 2: A_{280} nm comparison of the pooled bound and unbound fractions collected from purification of an unclarified *P. angolensis* seed lectin on fetuin-cryogel matrices.

Volume Loaded (ml)	A_{280} Bound pool	A_{280} Unbound pool
0.05	0.066	0.27
0.1	0.067	0.46
0.5	0.063	0.75
2	0.065	8.64

4.4 Improvement of the capacity of the cryogel based affinity matrix for *P. angolensis* seed lectin

The fetuin-cryogel matrix showed a low capacity for the crude homogenate independent of the volumes loaded to the cryogel matrix. The matrices were then subjected to several modifications of the method used by Kumar *et al.* (2003). The modifications included an extended coupling done twice and by also increasing the incubation time of the crude homogenates within the matrix. *P. angolensis* crude extract (2 ml) was fractionated into two distinct peaks as shown by protein assays (A_{280}) (Figure 8). The chromatographs showed higher lectin peaks, which revealed highly concentrated protein band on an SDS-PAGE (Figure 9). It was also evident on the gels that the binding of the lectin to the fetuin cryogel matrix was increased with an increased incubation time. This was also supported by the A_{280} nm readings of the bound pool (Table 3).

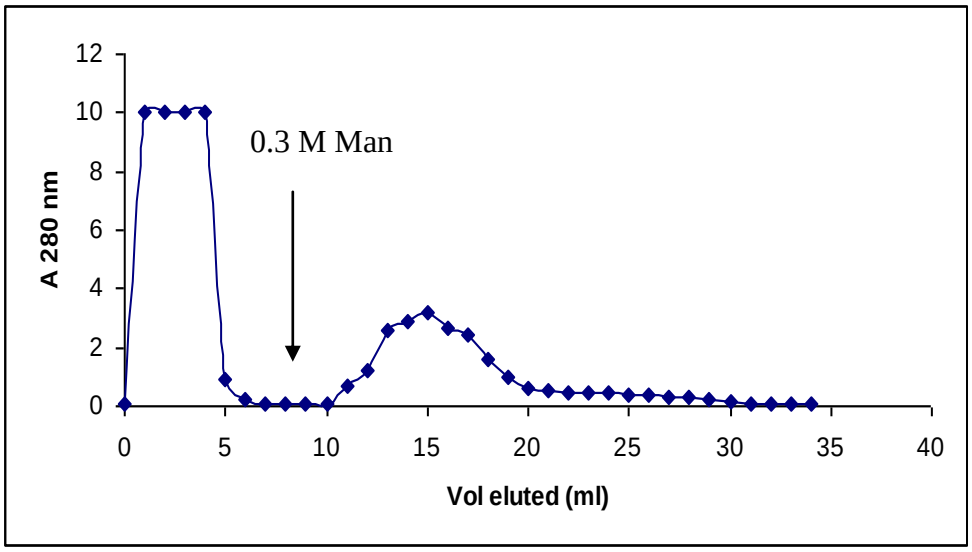


Figure 8: Elution profiles of *P. angolensis* seed lectin on fetuin supermacroporous affinity columns. *P. angolensis* crude extract (2 ml) was loaded on the modified fetuin pAAm-cryogels and incubated within the matrix for 1 h. Protein elution was monitored by measuring absorbance at 280 nm. The arrow indicates the specific buffer used to elute the lectin.

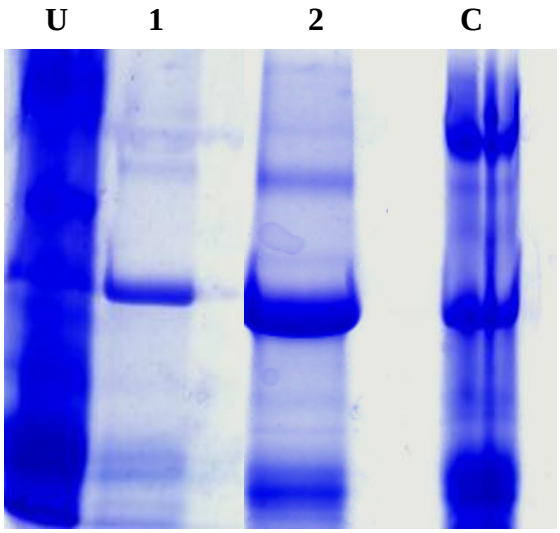


Figure 9: SDS-PAGE of purified lectin. Lane U: Unbound protein, lane 1: Bound pool of the 2ml loading onto fetuin pAAm-cryogel columns, lane 2: Bound pool of the 2 ml loading onto the improved set of fetuin pAAm-cryogel columns with an hour incubation within the matrices, lane C: Crude protein

Table 3: A $_{280}$ nm comparison of the pooled bound and unbound fractions collected from the purification of unclarified *P. angolensis* seed lectin on fetuin-cryogel matrices.

Set of Column and incubation time	***A_{280} Bound pool***	***A_{280} Unbound pool***
Cryogels with 1-step coupling, 15 min	0.1×10^3	1.5×10^3
Cryogels with 1-step coupling, 1 hr	0.9×10^3	2.8×10^3
Cryogels with an extended coupling, 15 min	0.1×10^3	1.0×10^4
Cryogels with an extended coupling, 1 hr	3.3×10^3	1.0×10^4

ELISA was done in order to obtain an elution profile that is specific for the lectin from fractions collected after purifying the unclarified crude extract of *P. angolensis* seed on the fetuin-cryogel matrix. The lectin fractions obtained when the crude extract was incubated for an hour within the modified fetuin-cryogel matrices had the highest reading at A_{405} nm (Figure 10a) compared with the readings when incubated for 15 min (Figures 10 b, c and d) on the same column or on the normal cryogels without an extended coupling, which has shown practically same results

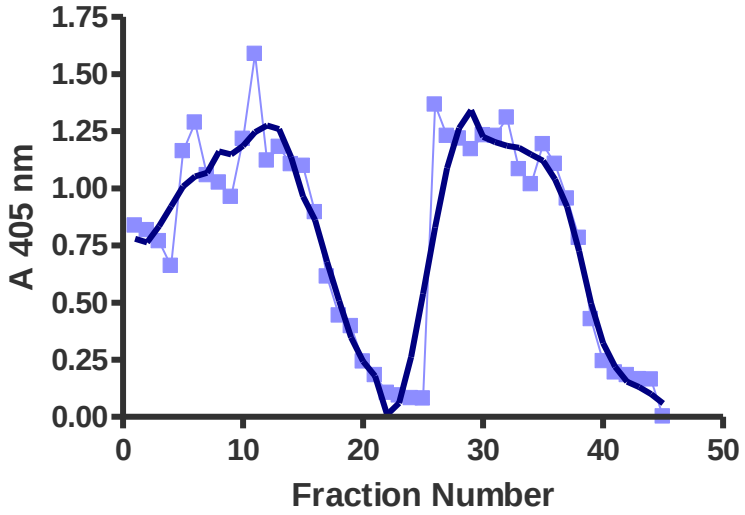


Figure 10a: The elution profile of *P. angolensis* lectin fractions purified on fetuin pAAm-cryogel. The crude homogenate (2 ml) was incubated for 1 h on a fetuin pAAm-cryogel with extended coupling.

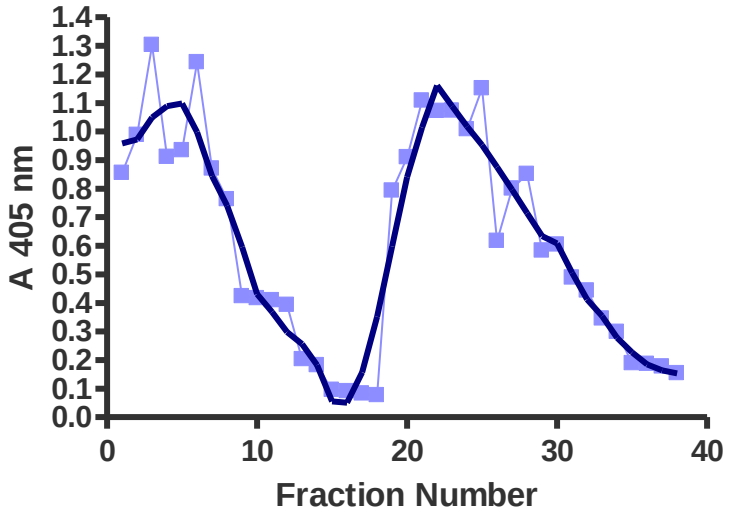


Figure 10b: The elution profile of *P. angolensis* lectin fractions purified on fetuin pAAm-cryogel. The crude homogenate (2 ml) was incubated for 15 min on a fetuin pAAm-cryogel with extended coupling.

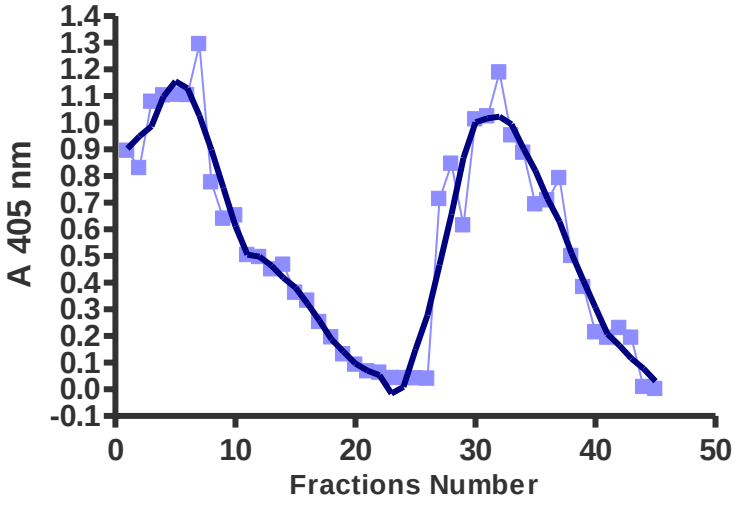


Fig 10c: The elution profile of *P. angolensis* lectin fractions purified on fetuin pAAm-cryogel. The crude homogenate (2 ml) was incubated for 1 h on a normal fetuin pAAm-cryogel.

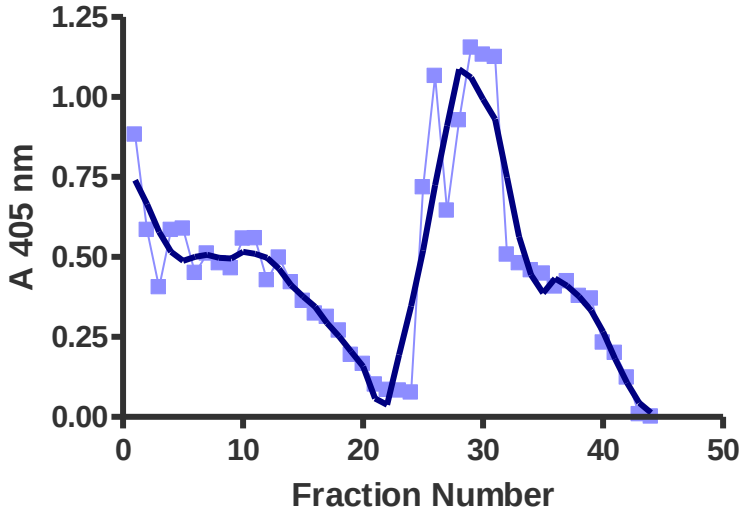


Figure 10d: The elution profile of *P. angolensis* lectin fractions purified on fetuin pAAm-cryogel. The crude homogenate (2 ml) was incubated for 15 min on a normal fetuin pAAm-cryogel.

4.5 Bioassays on cells treated with lithium chloride and actinomycin D to induce apoptosis.

4.5.1 Antiproliferative activity of lithium chloride and actinomycin D

The cytotoxicity effects of lithium chloride and actinomycin D at various concentrations on HL-60 cell lines were evaluated by a standardized trypan blue dye exclusion assay. Lithium chloride and actinomycin D showed a significant inhibition in proliferation of HL-60 cell cultures in a dose and time dependent manner (Figures 11a and b). Compared with the control, cultures incubated with 20 mM lithium and 5 µg/ml actinomycin D caused a progressive inhibition of growth within 48 h and 12 h respectively. These results were confirmed by cell viability assay where the results show that lithium chloride and actinomycin D not only inhibited the proliferation of the treated cell cultures but was also able to gradually decrease the percentage viability (Figures 12a and b) of the cells to about 40 % for actinomycin D treated within 12 h and to about 60 % for the lithium treated cells within 48 h.

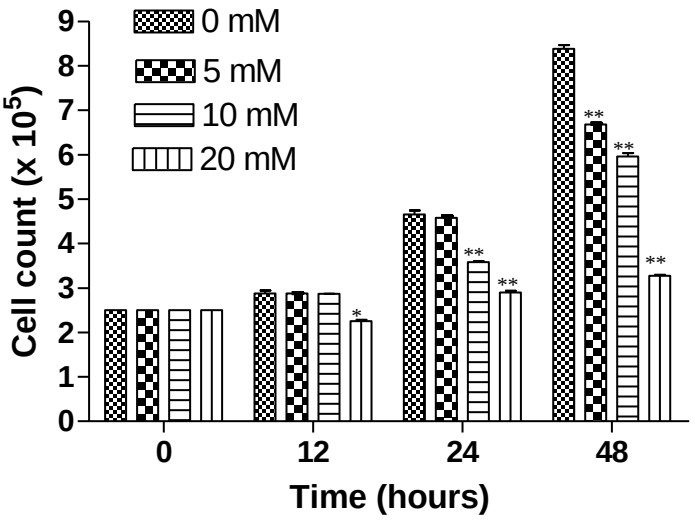


Figure 11a: The effect of lithium on the growth of HL-60 cells. Cells were cultured with or without different concentrations (0-20 mM) of lithium chloride for 12, 24 and 48 h. Each data represents the mean± S. D of three independent experiments. *p< 0.05, **p< 0.01.

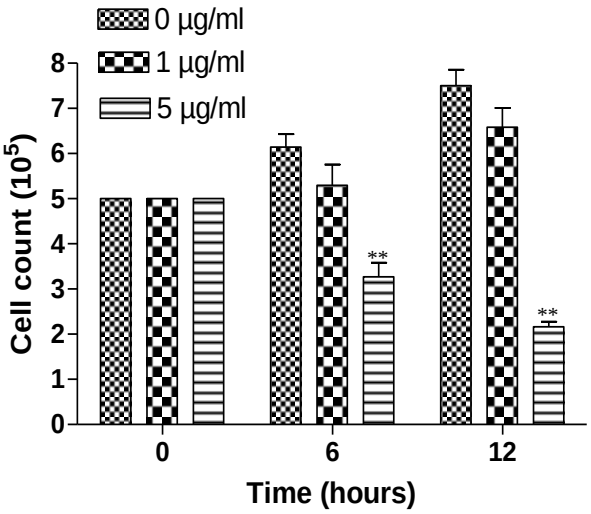


Figure 11b: The effect of actinomycin D on the growth of HL-60 cells. Cells were cultured with or without different concentrations (0-5 µg/ml) of actinomycin D for 6 and 12 h. Each data represents the mean± S. D of three independent experiments. *p< 0.05, **p< 0.01.

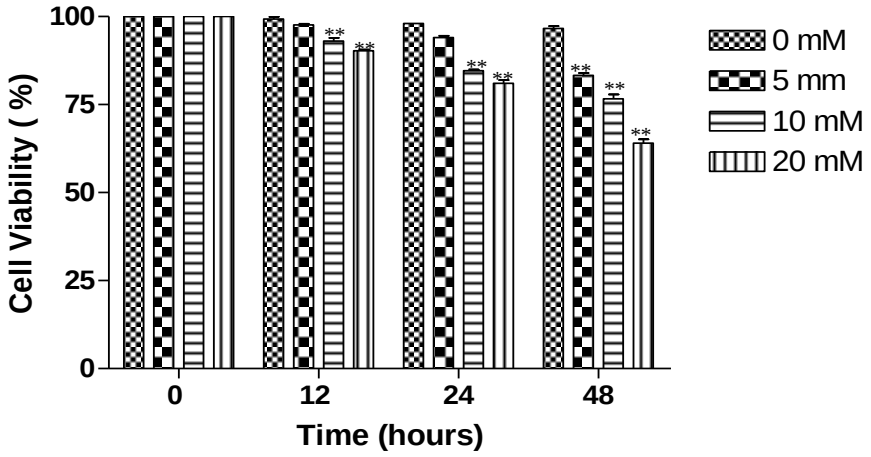


Figure 12a: The effect of lithium on the viability of HL-60 cells. Cells were cultured with or without different concentrations (0-20 mM) of lithium chloride for 12, 24 and 48 h, and the cell viability was determined using the trypan blue dye exclusion assay. Each data represents the mean± S. D of three independent experiments. *p< 0.05, **p< 0.01.

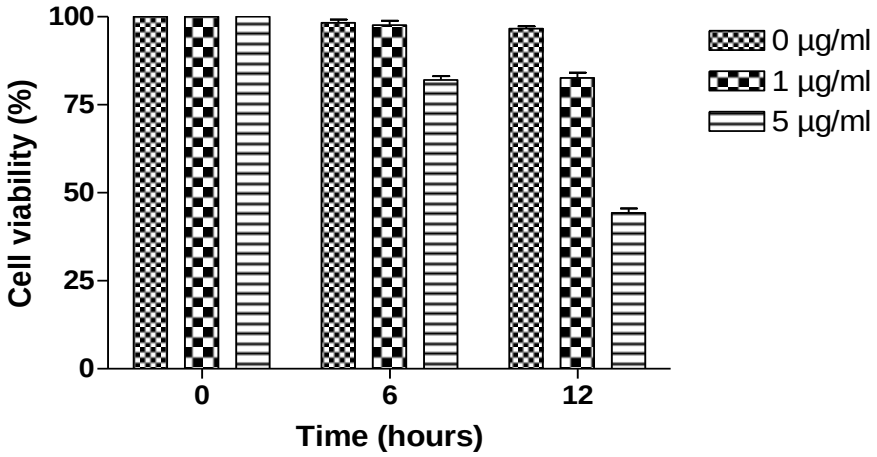


Figure 12b: The effect of actinomycin D on the viability of HL-60 cells. Cells were cultured with or without different concentrations (0-5 µg/ml) of actinomycin D for 6 and 12 h, and the viability was determined using trypan blue dye exclusion assay. Each data represents the mean± S. D of three independent experiments. *p< 0.05, **p< 0.01.

4.5.2 Analysis of apoptosis by DNA fragmentation.

Apoptosis of HL-60 cells were confirmed by detection of the integrity of DNA from treated cells or the fragmentation of chromosomal DNA with the classic DNA ladder method. A time dependent appearance of low molecular weight DNA ladder was observed in both the lithium treated HL-60 cells (Figure 13a) and actinomycin D treated cells (Figure 13b) compared to untreated cells which had only the high molecular DNA (Figure 13c). The results show DNA fragmentation characterized by oligonucleosomal fragments, a well-known feature indicative of programmed cell death (Goossens *et al*., 2000; Jin *et al*., 2006)

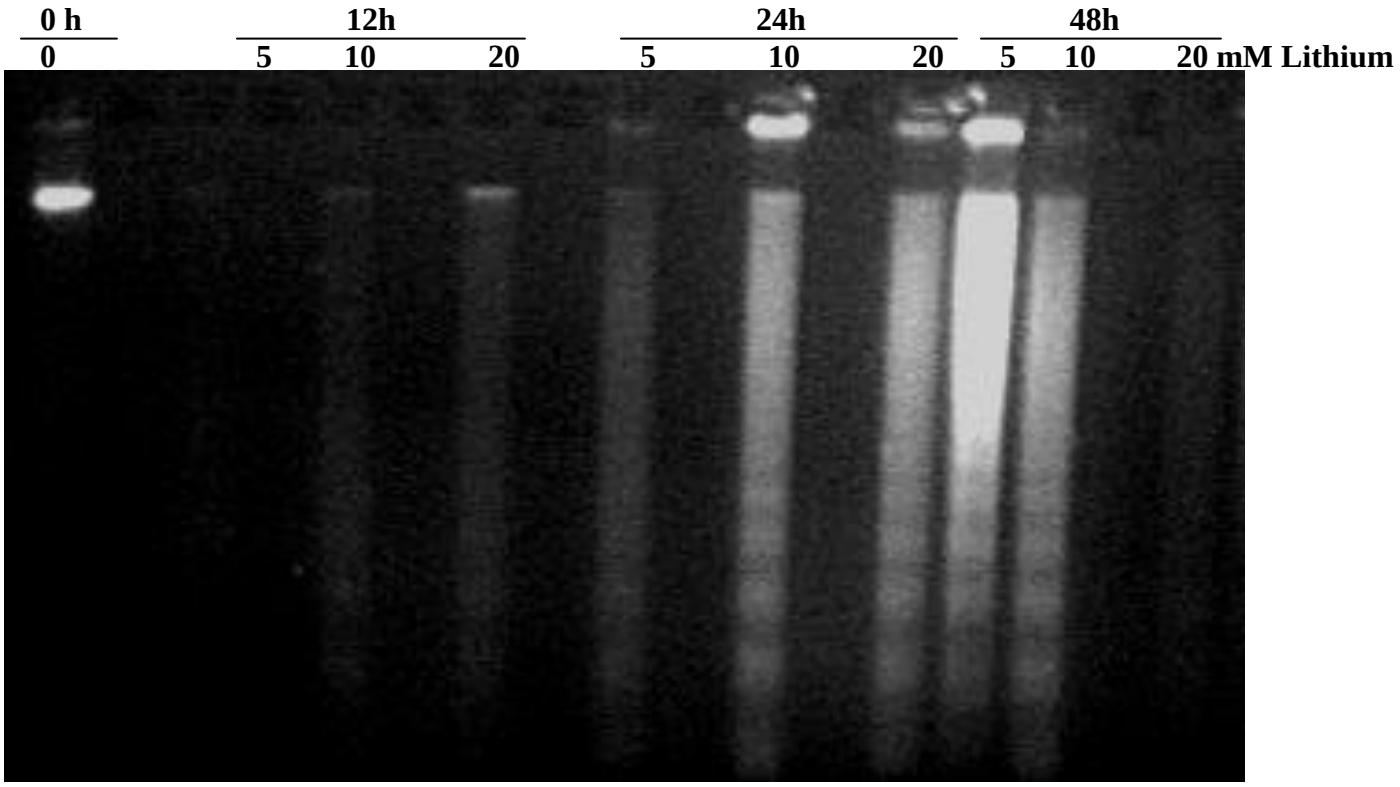


Fig 13a: Effects of various concentrations of lithium on HL-60 cell DNA after incubation periods of 0-72 h. The internucleosomal fragments were analyzed on a 1.5 % agarose gel. The low molecular weight DNA is contained within the supernatant after lysing the cells.

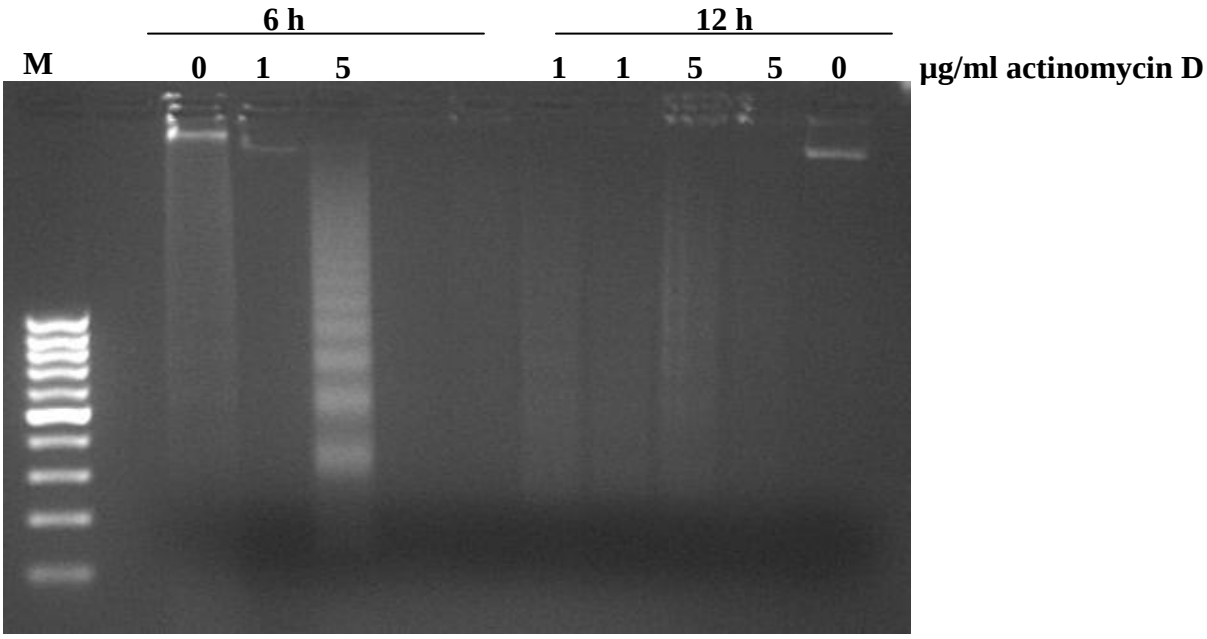


Figure 13b: Effects of various concentrations of actinomycin D on HL-60 cell DNA at different time intervals of 0-12 h. The internucleosomal fragments were analyzed on a 1.5 % agarose gel. The low molecular weight DNA is contained within the supernatant after lysing the cells

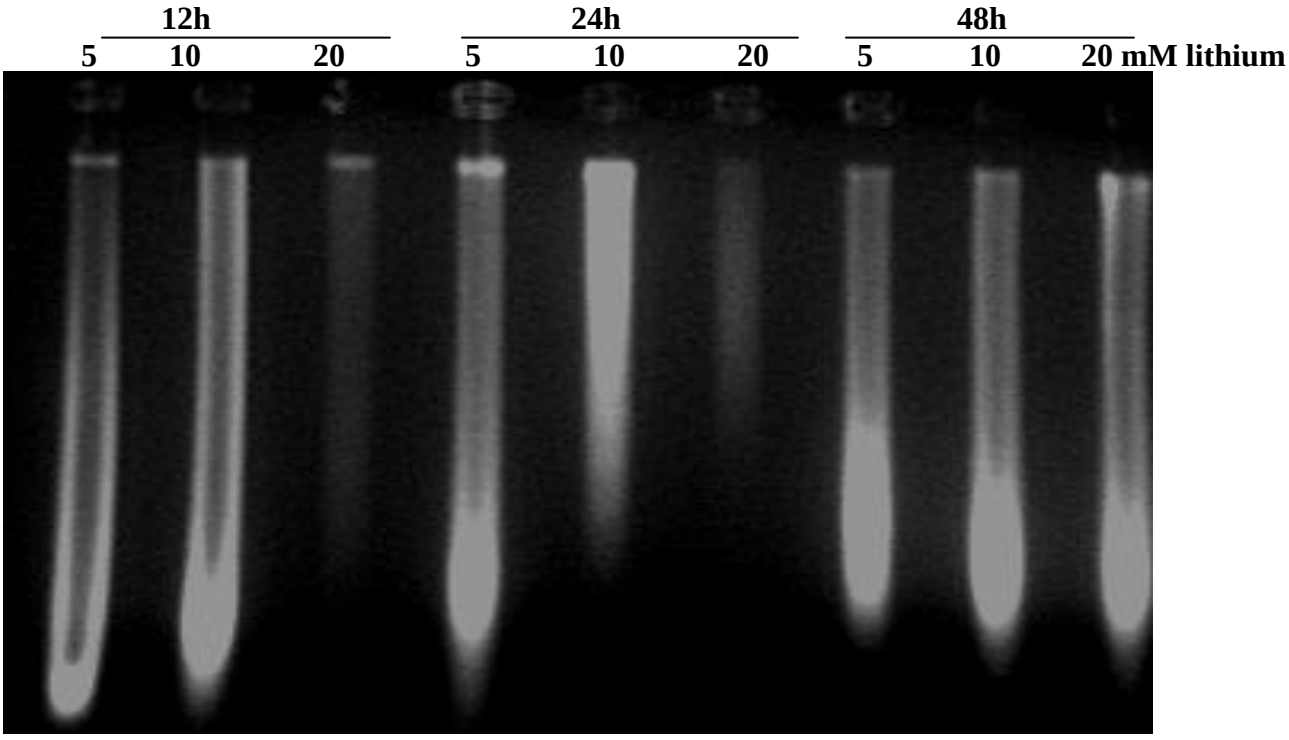


Figure 13c: Effects of various concentrations of lithium on HL-60 cell DNA at different time intervals of 0-72 h. The internucleosomal fragments were analyzed on a 1.5 % agarose gel. The high molecular weight DNA is contained within the pellet after lysing the cells.

4.5.3 Detection of early stages of apoptosis using annexin-V

Annexin-V staining permits the detection of phosphatidylserine exposure on the outer layer of membranes of apoptotic cells. The cells treated with different concentrations of lithium for 6 and 24 h time intervals were stained with annexin V-FITC and propidium iodide in order to detect the morphological indicators of early stages of apoptosis. The cells treated with different lithium concentrations preferentially bind annexin V-FITC within 6 h of culturing, showing the most binding with 5 mM and 10 mM of lithium treatment as compared with 20 mM lithium treatment as shown by cells staining positive with annexin V-FITC (Figure 14a), which excluded propidium iodide. The cells later (at 24 h) stained positive for both annexin V-FITC and propidium iodide (Figure 14b).

The translocation of phosphatidylserine to the outer site of the membranes was also shown on membranes of HL-60 cells treated with actinomycin D within 2 – 4 h. The fluorescent readings showed maximum binding within 2 h (Figure 15a), which was supported by fluorescent microscopic pictures (Figure 15b). The results also show a higher binding capacity for annexin V-FITC on actinomycin treated cells as compared with the lithium treated cells.

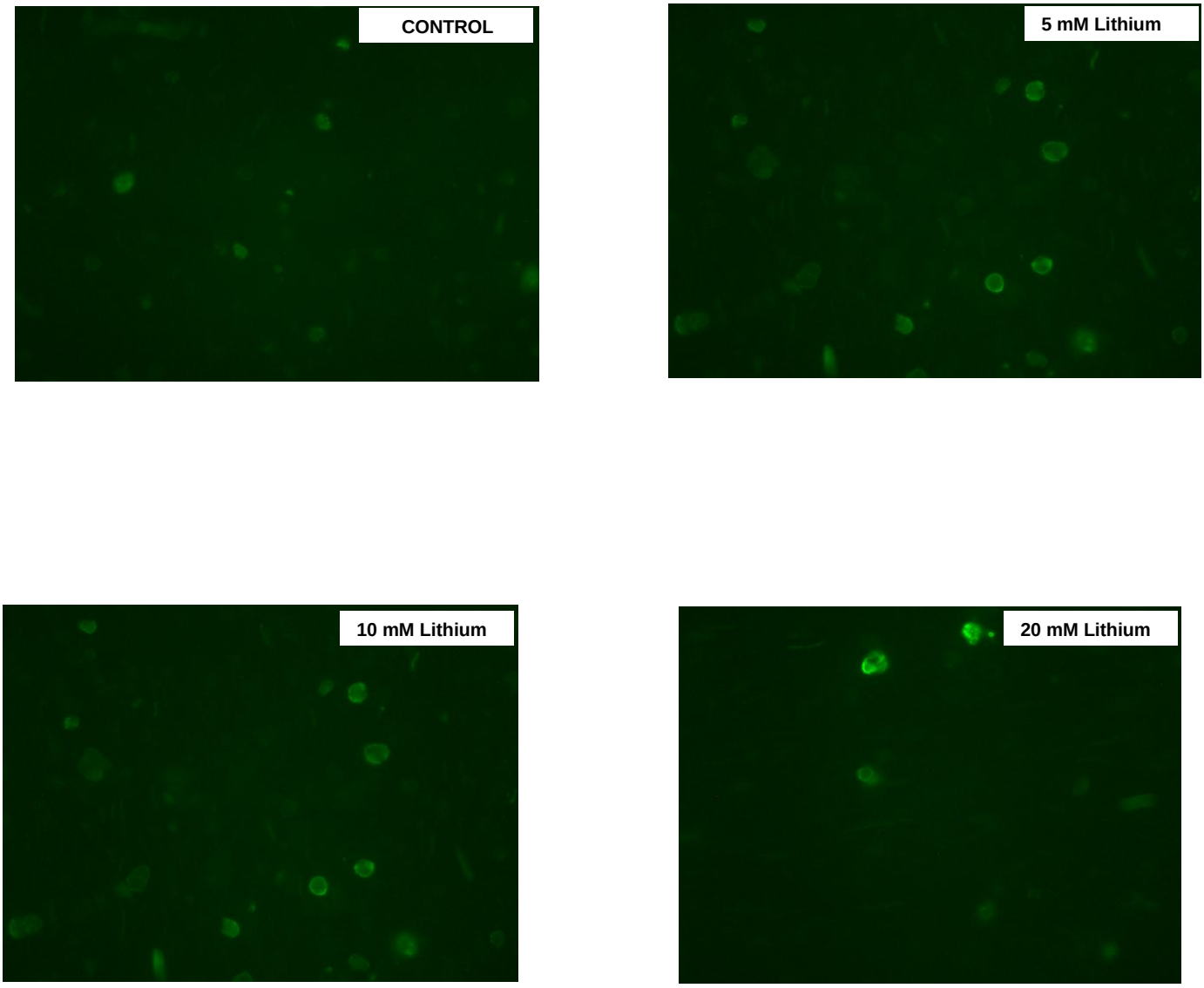


Figure 14a: Microscopic analysis (40 x magnification) of annexin V-FITC stained HL-60 cells after 6 h incubation with 5 mM, 10 mM and 20 mM lithium.

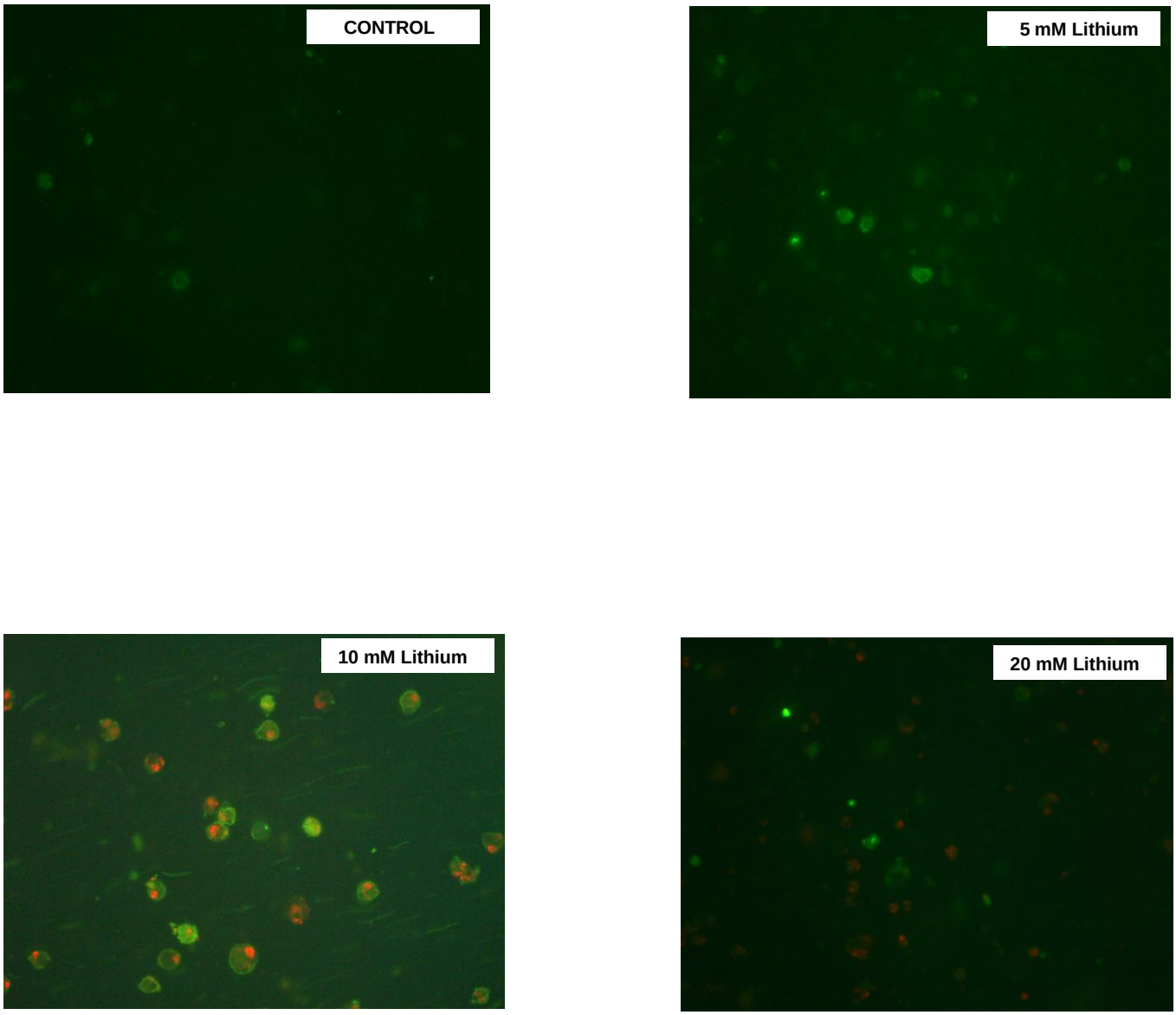


Figure 14b: Microscopic analysis (40 x magnification) of annexin V-FITC stained HL-60 cells after 24 h incubation with 5 mM, 10 mM and 20 mM lithium.

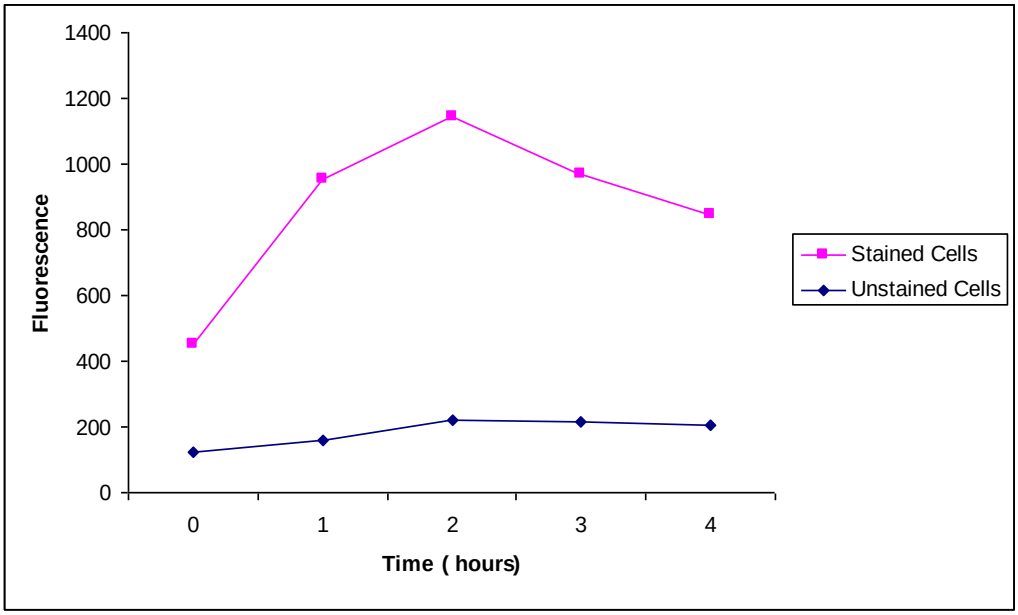


Figure 15a: Fluorescent readings of annexin V-FITC stained HL-60 cells. Cells were either treated or not treated with 5 µg/ml actinomycin D for 0-4 h.

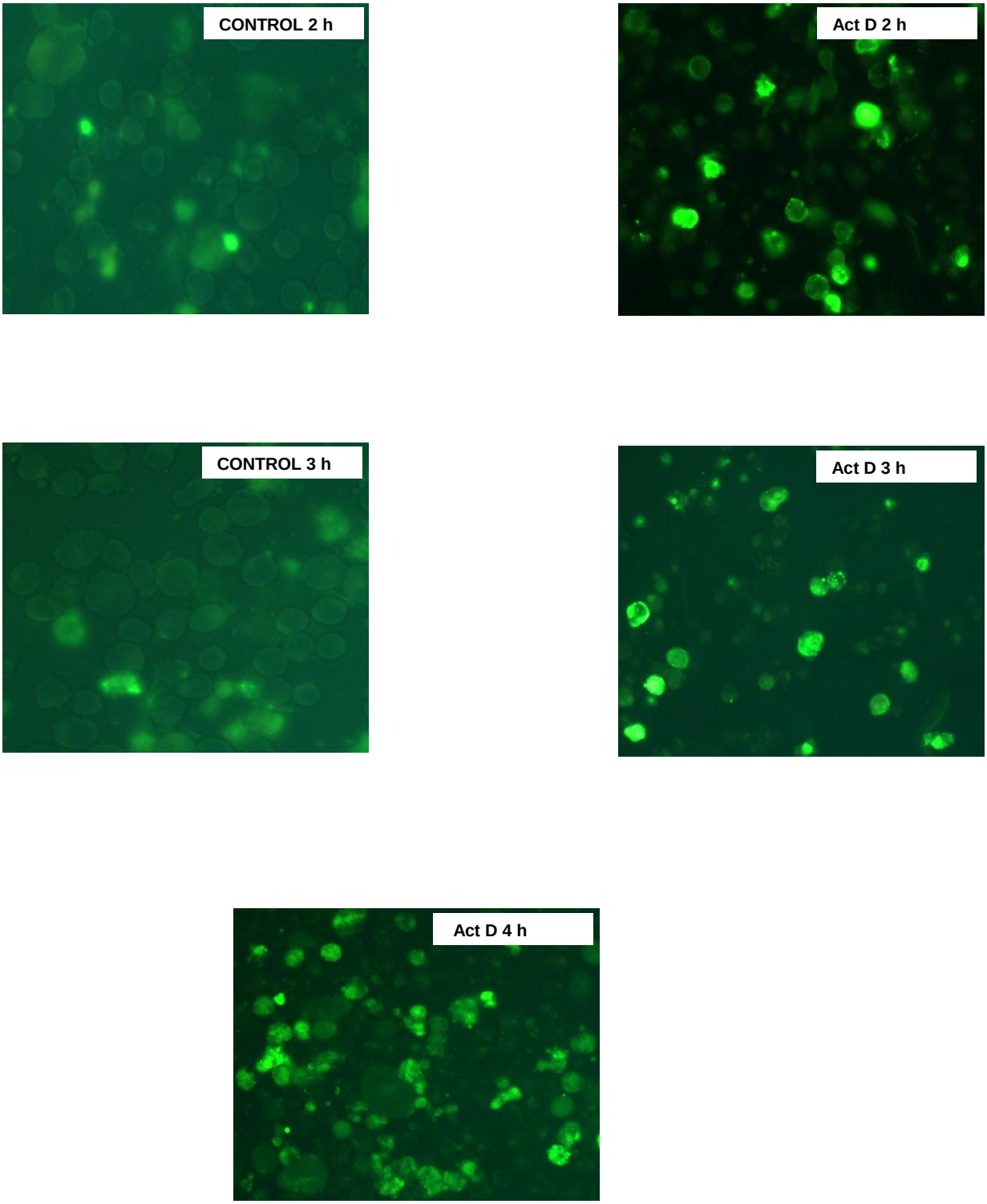


Figure 15b: Microscopic analysis (40 x magnification) of annexin V-FITC stained HL-60 cells. Cells were either treated or not treated with 5 µg/ml actinomycin D over 0-4 h.

4.5.4 Effects of actinomycin D on the expression of Bax and BCl-2.

In order to study the positive involvements of Bcl-2 and Bax (respectively, the anti-apoptotic and pro-apoptotic proteins of the Bcl-2 family) in regulating apoptosis, cells were treated with 5 µg/ml actinomycin D for 2- 6 h. The western blot analysis of Bcl-2 and Bax proteins showed that actinomycin D down-regulated Bcl-2 levels and up-regulated Bax levels (Figure 16a). The highest down regulation of Bcl-2 was achieved when cells were treated for 2 h whilst the most up-regulation of Bax was achieved at 4 h. The densitometric band analysis was subsequently done to support the observed down-regulation of Bcl-2 and up-regulation of Bax proteins (Figure 16b).

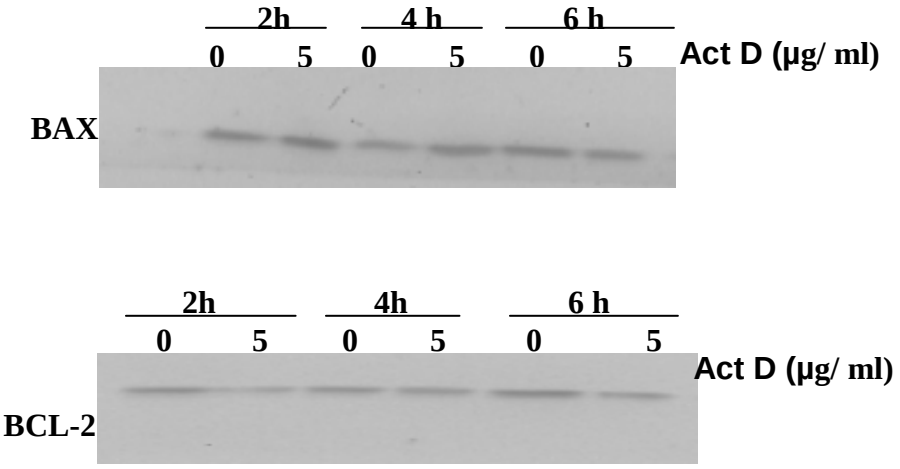


Figure 16a: Effects of Bcl-2 family protein expression of HL-60 cells. Bcl-2 and Bax were examined by Western blotting of protein extracts of HL-60 cells either treated or not treated with 5 µg/ml actinomycin D for 2-6 h.

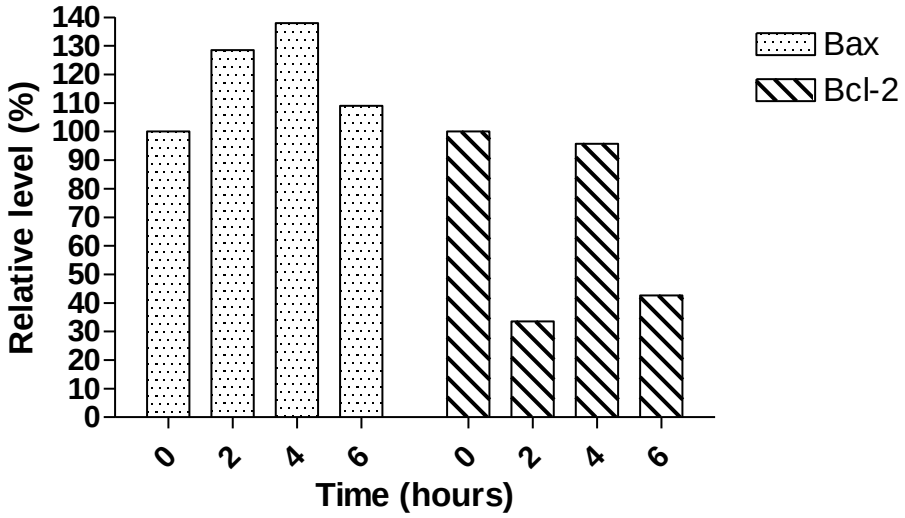


Figure 16b: Relative expression levels of the Bax and Bcl-2 proteins. Bcl-2 and Bax were examined by western blotting of protein extracts of HL-60 cells untreated or treated with 5 µg/ml actinomycin D for 2-6 h.

4.6 Lectin binding studies

4.6.1 Screening of lectins as biochemical markers of apoptosis

Several lectins (*Pterocarpus angolensis*, Concanavalin A, Red kidney bean, *Pisum savitum*, *Tritum vulgaris*, and *Helix pomatia*) were screened for use as new biochemical markers of apoptosis that could be easily used for analysis without the need for cell damage. The lectins used were able to bind preferentially on the cell membranes. The fluorescent readings showed that the *T. vulgaris* lectin had a high binding capacity for the sugars that were expressed during apoptosis as seen on Figures 17a and 17b, compared with the other lectins used. These findings were supported by the fluorescent microscopic pictures (Figure 18a), which also revealed that the HL-60 cells treated with actinomycin D even able to stain the interior of the apoptotic cells to some extent and further express particular antigens that are recognized by *T. vulgaris* lectin as observed by cells forming clumps compared with other lectins used (Figure 18b).

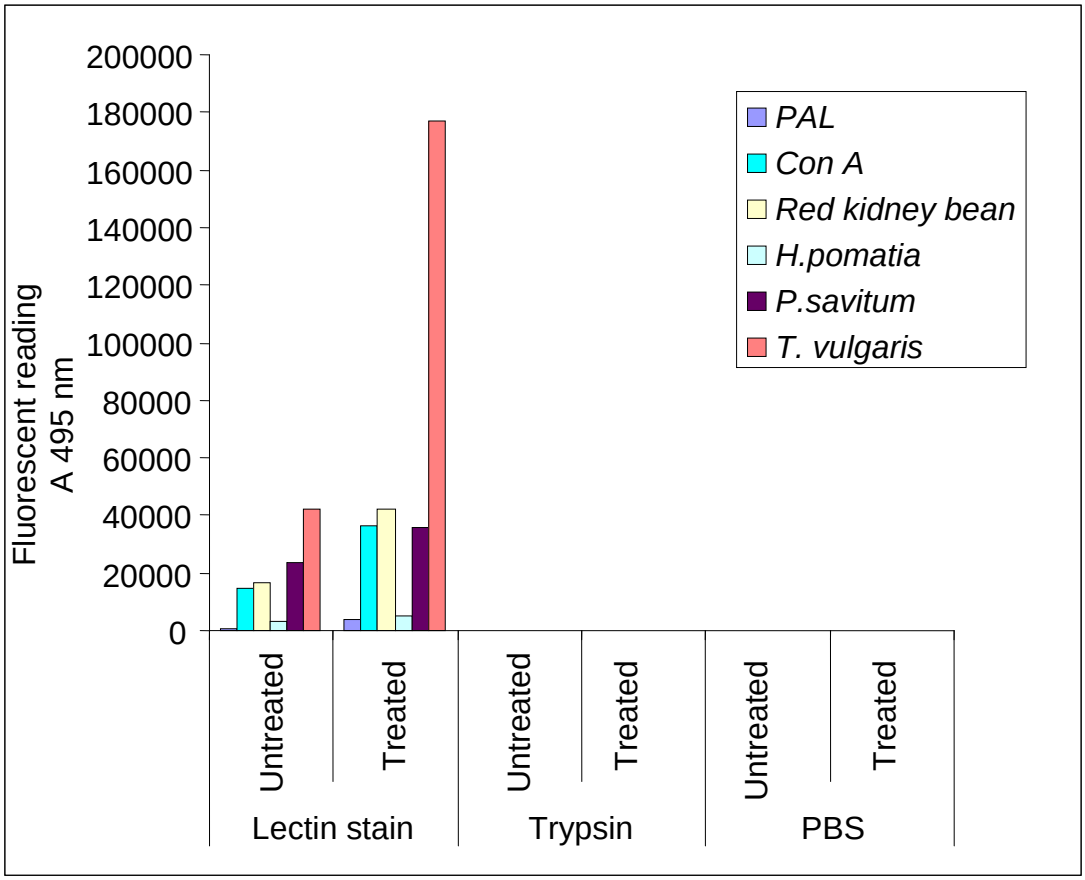


Figure 17a: Fluorescent readings of different FITC-labelled lectins on HL-60 cells. Cells were either treated or not treated with 5 µg/ml actinomycin D for 2 h and stained with different lectins or trypsin and PBS.

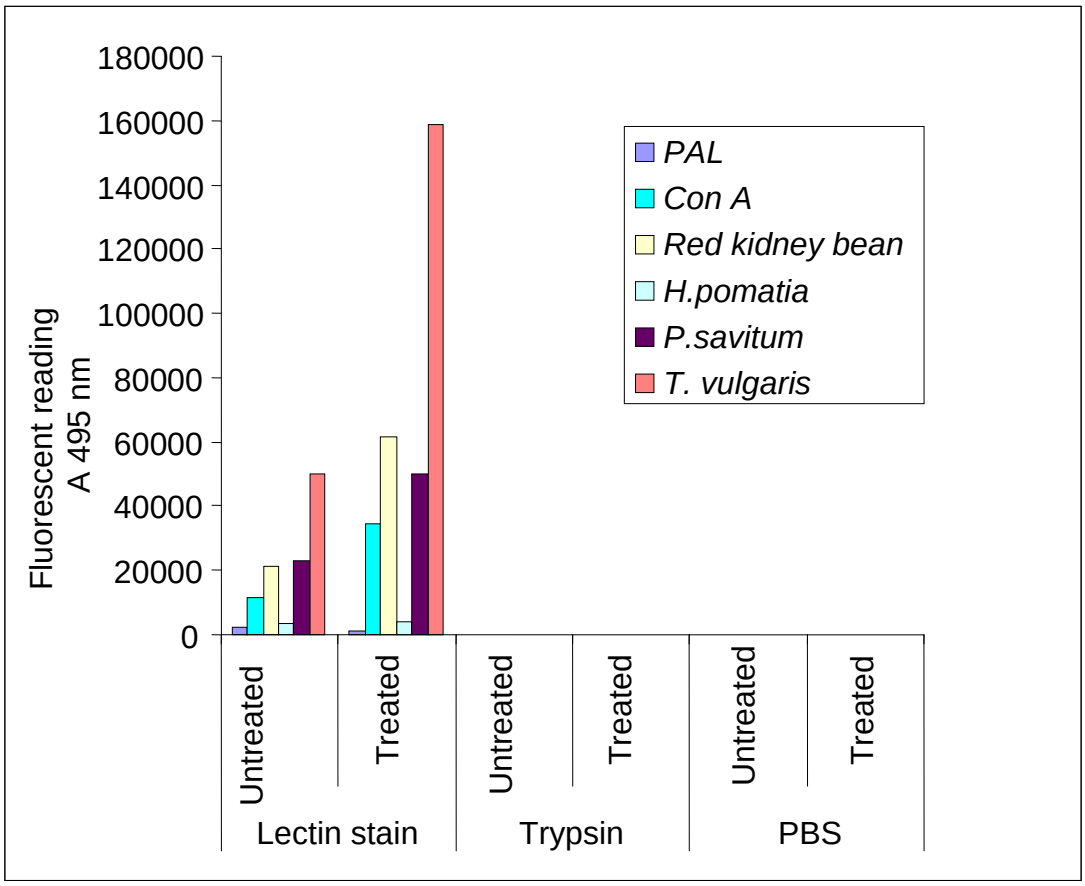
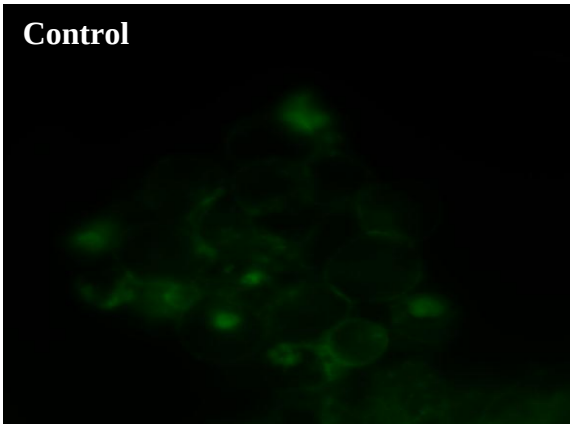
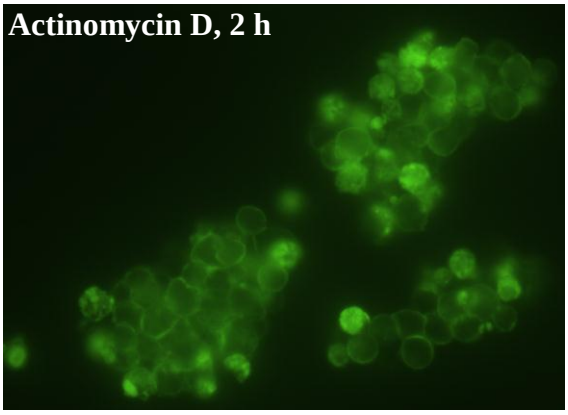


Figure 17b: Fluorescent readings of different FITC-labelled lectins on HL-60 cells. Cells were either treated or not treated with 5 µg/ml actinomycin D for 3 h and stained with different lectins of trypsin and PBS.





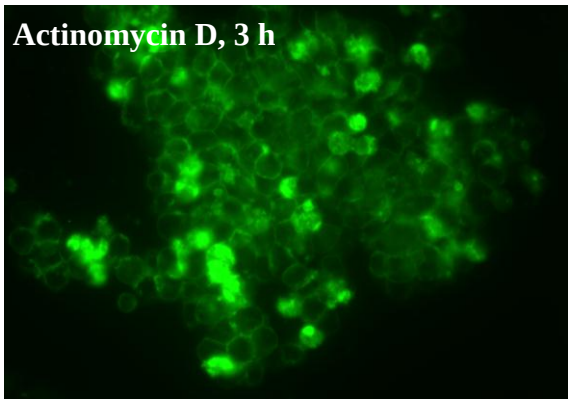
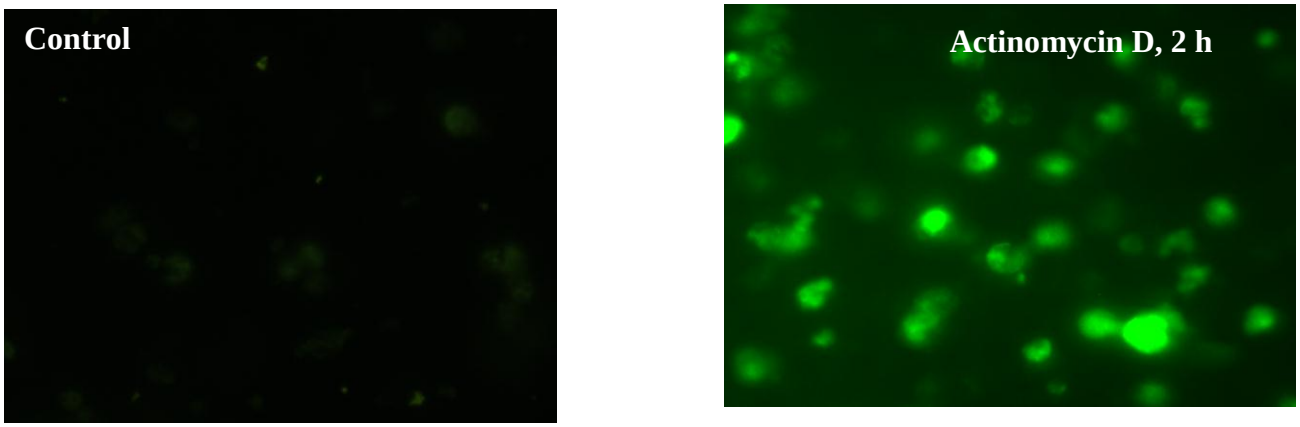


Figure 18a: Microscopic analysis (40 x magnification) of FITC-*T. vulgaris* stained HL-60 cells. Cells were either treated or not treated with 5 µg/ml actinomycin D for 2 and 3 h.

A



B

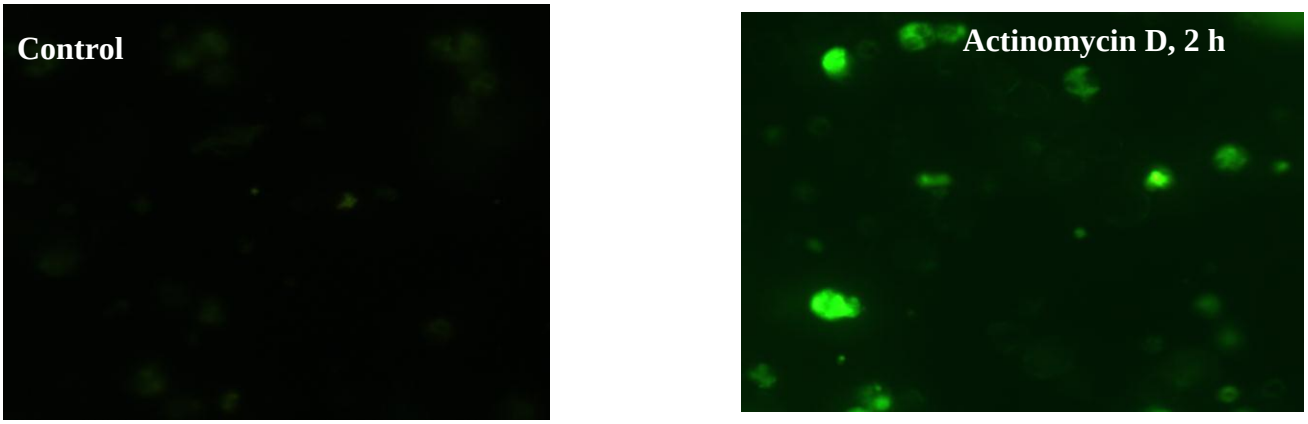


Fig 18b: Microscopic analyses (40 x magnification) of FITC-lectin stained HL-60 cells. Cells were either treated or not treated with 5 µg/ml actinomycin D for 2 h and stained with (A) FITC-Red kidney bean and (B) FITC- Con A.

4.6.2 Titration of *T.vulgaris* lectin

In order to estimate the lectin-binding capacity of the cells, increasing amounts of fluorescent labelled *T. vulgaris* lectin were allowed to interact with a fixed number of cells. Upon incubation with increasing amounts of lectin with the actinomycin D treated cells, the fluorescent intensity was observed to increase with an increase in the concentration of the lectin (Figure 19).

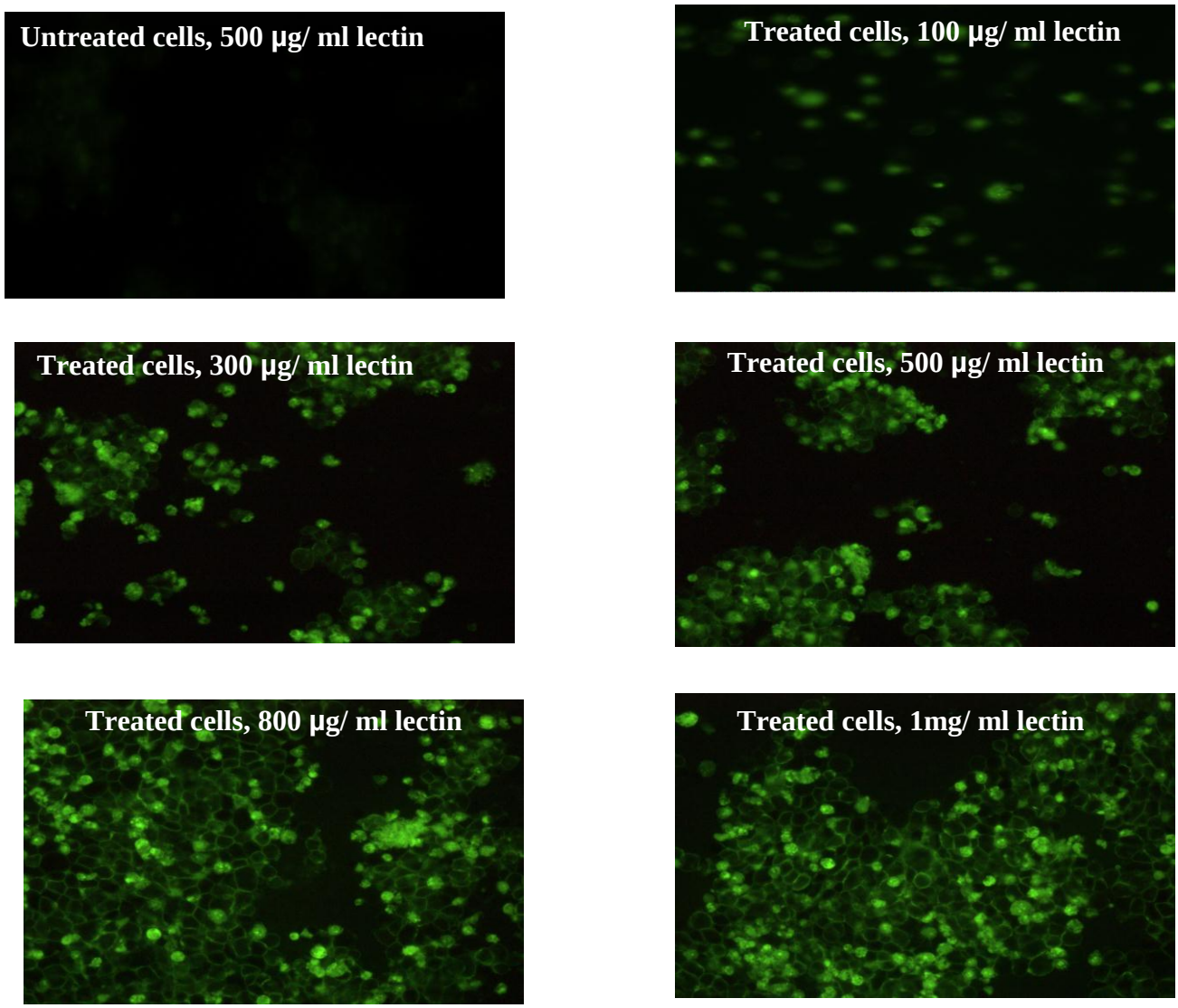
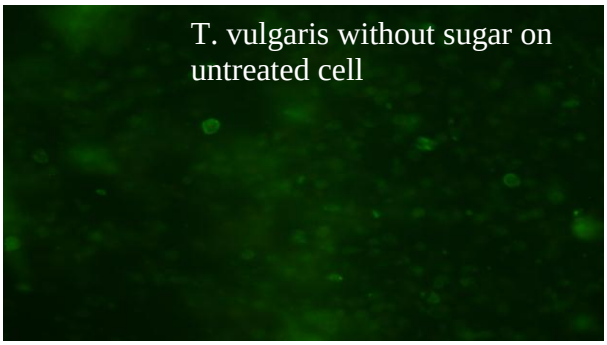


Figure 19: Microscopic analysis (40 x magnification) of FITC-lectin stained HL-60 cells. Cells were not treated or treated with 5 µg/ml actinomycin D for 2 h and stained with different concentrations (100 µg/ml- 1 mg/ml) of FITC-*T. vulgaris* lectin.

4.6.3 Sugar inhibition tests

In order to evaluate the specificity of *T.vulgaris* lectin to the apoptotic, the cells were incubated with a lectin with BSA, a lectin with PBS and a lectin with N-acetyl glucosamine. Untreated cells stained by *T. vulgaris* lectin and incubated with sugar did not show any specific binding on the membrane and neither did they form clumps under the fluorescent microscope (Figure 20). Treated cells were incubated with PBS and BSA together with the *T.vulgaris* lectin, showed membrane binding of the lectin when observed under the fluorescent microscope compared with treated cells that were incubated with a specific sugar which did not show specific interaction with the membrane glycoproteins nor formed clumps of the apoptotic cells (Figure 21). This is a result of the sugar competing with the membrane glycoproteins for binding to the lectin.



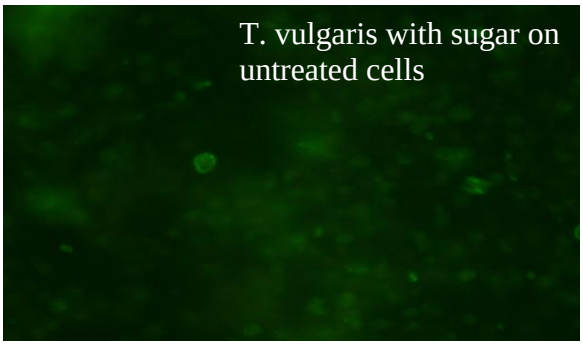


Fig 20: Microscopic analysis (40 x magnification) of FITC-lectin stained HL-60 cells. Cells were not treated with 5 µg/ml actinomycin D and stained with 500 µg/ml of FITC-*T. vulgaris* lectin pre-incubated with 0.2 M N-acetyl glucosamine.

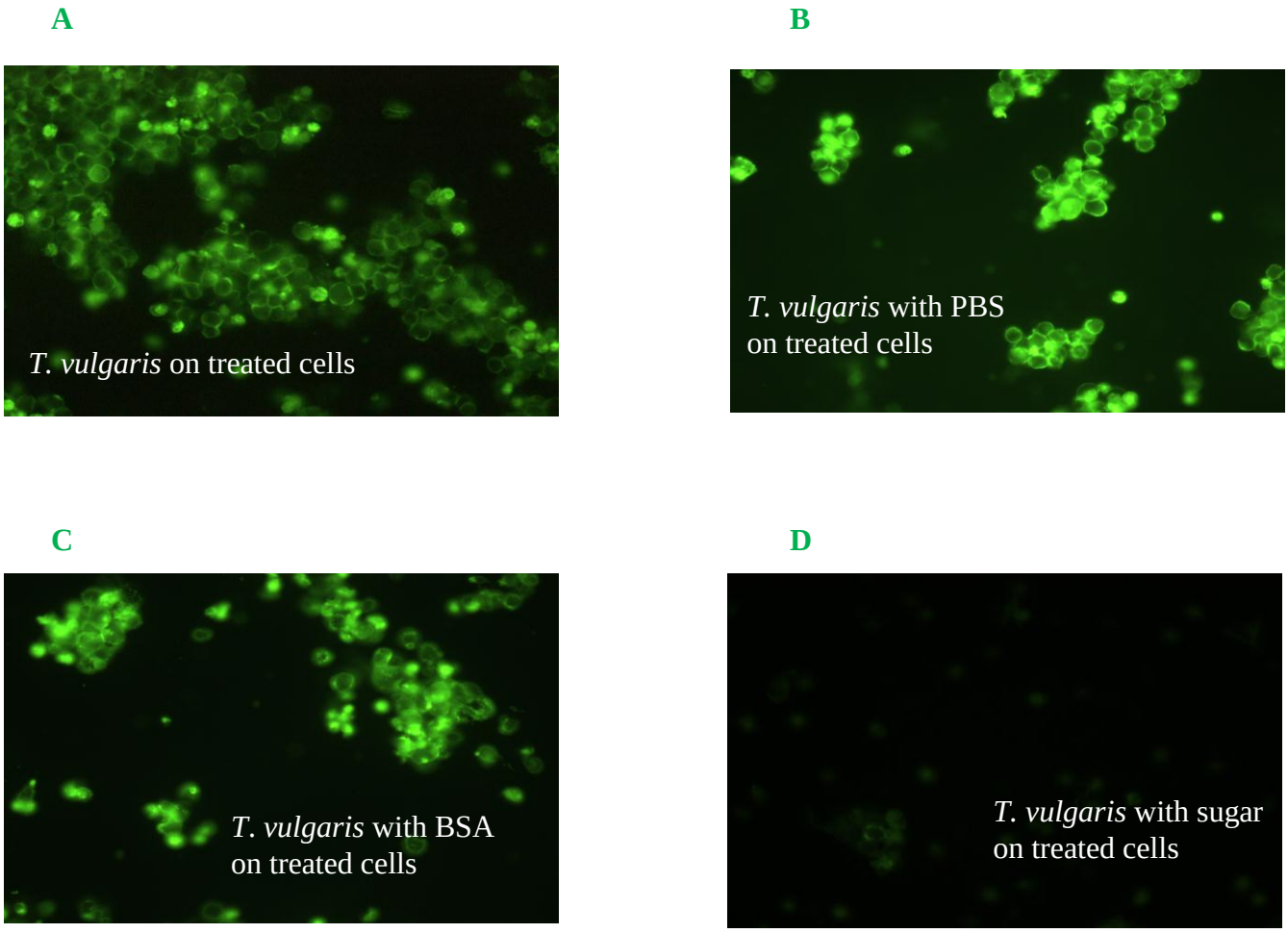


Fig 21: Microscopic analysis (40 x magnification) of FITC-lectin stained HL-60 cells. Cells were treated with 5 µg/ml actinomycin D and stained with (A) 500 µg/ml of FITC-*T. vulgaris* lectin, (B) 500 µg/ml of FITC-*T. vulgaris* lectin pre-incubated with PBS, (C) 500 µg/ml of FITC-*T. vulgaris* lectin pre-incubated with BSA and (D) 500 µg/ml of FITC-*T. vulgaris* lectin pre-incubated with N-acetyl glucosamine.

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Purification of *P. angolensis* seed lectin using Sephadex G-75 affinity chromatography

An increasing use of plant lectins in chemical and biological research has prompted the development of many methods for their purification (Oliveira *et al*., 2002). This study was designed to purify plant lectins and later use them to probe the differences between the membranes of cancerous cells and those that are undergoing apoptosis. Because of its specificity, affinity chromatography has been used most widely, and several types of ligands and support matrices have been used (Bakalova and Ohba, 2002). The lectin from the *P. angolensis* seed extract was separated into three major peaks (Figure 3). The first and second peaks eluted as high broader peaks, indicating the presence of two close protein peaks, whereas the lectin peak was retarded within the column. It seems that binding of the lectin to the Sephadex G-75 column is not strong, thus lectin is retarded, and elutes at a later stage without a buffer change.

Sephadex is a dextran polymer of glucose units. Since the *P. angolensis* seed lectin is a mannose and glucose specific, this offers the opportunity to purify the lectin through weak binding of the lectin to the Sephadex G-75 column. It was evident that all the lectin fractions collected within the peak shown in Figure 3, contained a homogenous pure protein as noticed by single bands of approximately 28 kDa (Figure 4). This is the known subunit molecular weight of *P. angolensis* seed lectin (Beeckmans *et al*., 2002) which also correlates with the studies done by Iams (1999), that all the *Pterocarpus* lectins consist of a 28 kDa subunit that is stable over a wide range of pH values and very resistant to denaturation.

Traditional approaches for the purification of *P. angolensis* seed lectin on Sephadex G-75 is quite a promising preparative scale technique, but limited by scale, time and several purification procedures e.g; several centrifugation steps and ammonium sulfate precipitation. pAAm monolithic cryogels have been used in this study to improve some of these limitations. These matrices have been recently developed and show a great promise in various biological applications. The monolithic cryogels typically have a system of interconnected macropores

allowing unhindered diffusion of solutes of practically any size (Arvidsson *et al*., 2002; Kumar *et al*., 2003) as well as mass transport of nano- and microparticles (Kumar *et al*., 2006).

5.2 Evaluation of glycoprotein-cryogel affinity column for purification of PAL from crude homogenates.

According to Beeckmans *et al*. (2002) fetuin was found to be the most effective glycoprotein that inhibits agglutination of erythrocytes by *P. angolensis* seed lectin. Based on these observations, the supermacroporous cryogels were coupled with fetuin through a spacer arm. The introduction of the spacer arm improves binding of the crude to the immobilized ligand. Single-step purification of an unclarified *P. angolensis* crude homogenate was achieved on these fetuin-cryogels. Similar elution profiles and SDS-PAGE gels were observed on all separate fetuin cryogels loaded with the crude extract ranging from 0.05 ml to 2 ml (Figure 6). The electrophoretic patterns of the bound fractions showed major bands and other light bands (Figure 7), which could be slight contamination of other proteins that were bound non-specifically to the gel matrix.

Data from the purification procedure represented in Table 1 have shown a higher yield achieved with the 0.5 ml loading with 5 fold purification as compared to other loadings. The lower purification fold could be due to the slight protein and non-protein contaminants that were entrapped non-specifically to the cryogel matrix resulting in a decreased purification factor. The cryogel matrix showed the same capacity for different volumes loaded (Table 2), i. e. the amount of lectin bound did not change with the volume loaded onto the column. The *P. angolensis* seed crude lectin is the major protein in the seed extract and known to have a higher concentration of the lectin (21.6 g) from 1 kg of dry seed (Echemendia-Blanco *et al*., 2009). The high concentration of *P. angolensis* seed lectin could have also contributed to the easy overloading and the capacity of the cryogel matrix for the lectin.

The results further suggest that the capacity of the cryogels was fully utilised even with small volumes as little as 0.5 ml that are loaded to the cryogels. The low capacity of the cryogel matrix for the protein could be due the existence of relatively low coupling sites of the gel matrix. These cryogel matrices have a polymer of hydroxyl groups that could have resulted in the lower ligand density. The lower ligand density attributed to the lower adsorption or

desorption events for the lectin that could have resulted in the lower selectivity of the column and hence the non-specific binding to the matrices. The decrease in capacity could be compensated by preparing cryogel adsorbents with increased ligand density.

Optimisation was done with respect to the ligand density and an increased incubation time. The pAAm-epoxy containing supermacroporous matrices coupled with fetuin through extension of the spacer arm with a two-step derivatisation that includes reaction with ethylenediamine showed an increased binding of the lectin to the fetuin-pAAm cryogel. It was further assumed that binding of the unclarified crude would be increased by multipoint interactions to the ligand and an increased incubation time (for 1 h compared with 15 min) (Figure 9). ELISA assays were done in order to detect and quantify the amount of *P. angolensis* seed lectin, but could not detect the differences observed on the gels due to high concentrations of the lectin. It is important to note that application of the unclarified feed at maximum flow rates did not cause compression or any kind of deformation of the cryogel matrix even after several repetitive runs; the columns did not demonstrate any deterioration in performance. No back pressure occurred when the unclarified crude of *P. angolensis* seed was passed through the column at a flow rate of 1 ml/min, indicating that the crude extract passed through the channels of the cryogel matrix without being trapped.

5.3 Screening the several stages of apoptosis

Another approach of this study was to investigate the use of plant lectins as novel plasma membrane markers that binds specifically to the carbohydrate component of the glycoprotein molecule during programmed cell death. Reference was made to previous studies done by Bilyy and Stoika (2003) that specific plant lectins could recognise changes in structures of specific glycoconjugates which become accessible after treatment with potent inducers of apoptosis. Cytotoxicity studies provide important preliminary data to help select potential antineoplastic chemicals. Data from this study showed that treatment of HL-60 cells with lithium and actinomycin D resulted in a time- and dose-dependent inhibition of proliferation of HL-60 cells (Figures 11a and b). The results seem to suggest cell death due to treatment with lithium and actinomycin D. This induced tumour cell death confirms that both lithium and actinomycin D can be used as potential agents that induce apoptosis (Becker and Tyobeka, 1996; Goossens *et al*., 2000; Matsebatlela *et al*., 2000). The induced cell death was confirmed

by the cell viability assays where trypan blue dye was used to evaluate the cytotoxicity effects of lithium and actinomycin D on the viability of HL-60 cells. Lithium and actinomycin D not only inhibited the proliferation of the experimental cell cultures but were also able to decrease the percentage viability of the cell cultures in a time- and dose-dependent manner (Figures 12a and b). Our current results are consistent with previous studies that are well documented, which indicate that lithium at concentrations of 10 mM and above inhibits cell growth of HL-60 promyelocytic leukemia cells and that the induced cell death is due to apoptosis (Adams and Cory, 1998;Madiehe *et al*., 1995; Matsebatlela *et al*., 2000).

Since results obtained from cell proliferation and viability studies confirmed a varying degree of sensitivity to lithium and actinomycin D, further experiments were done to categorise different stages of apoptosis arising after the treatment, by investigating the effects of lithium and actinomycin D on cellular morphology and biochemical features indicative of apoptosis. The agents causing apoptotic cell death can be broadly classified as those producing DNA ladder as an early event (occurring within 6 h of treatment) and those in which DNA laddering occurs as a late event (requiring more than 24 h for cells to exhibit DNA cleavage) with the use of transcription and translation inhibitors (Madiehe *et al*., 1995). The DNA laddering technique was used to visualise the edonucleolytic cleavage of the products of apoptosis, which are indicators of the late stages of apoptosis. In this study, both lithium and actinomycin D induced DNA fragmentation in a time-dependent fashion (Figures 13a and b). More importantly, extensive DNA ladder was observed within six h of treatment of experimental cultures with actinomycin D (Figure 13b), whilst treatment of experimental cultures with lithium showed DNA ladder after 24 h. The occurrence of apoptosis as a late event suggest that lithium induced apoptosis requires protein synthesis. Alternatively, it might indicate the suppression of products responsible for protecting against apoptosis, for example Bcl-2 and myc genes (Hofseth *et al*., 2004).

In order to detect early morphological markers of apoptosis, annexin V-FITC was used to detect surface changes i.e., externalisation of phoshatidylserine residues. Annexin V-FITC staining precedes the loss of membrane integrity, which accompanies the latest stages of cell death resulting from apoptosis. Results from this study showed that lithium treated cells were undergoing early stages of apoptosis by binding to annexin V-FITC within 6 hours of incubation (Figure 14a). This data later (at 24 h) showed a positive staining for both annexin

V-FITC and propidium iodide, suggesting that the treated cells were already at a later stage of apoptosis and were already dead and the membrane is no longer intact, thus allowing permeability of propidium iodide. Our data also showed phosphatidylserine exposure on actinomycin D treated cells within 2-4 h of incubation (Figures 15a and b).

HL-60 cells demonstrated a varying degree of sensitivity to lithium and actinomycin D, with actinomycin D showing to be more potent than lithium, even at higher concentrations. Earlier studies have shown actinomycin D as a potent inducer of apoptosis in a variety of cells *in vitro* and *in vivo*, by binding to DNA and further inhibiting RNA and protein synthesis (Goossens *et al*., 2000; Taha *et al*., 2004). Despite the observations of actinomycin D, on a variety of cells, its effects on HL-60 cells have not been extensively investigated.

The Bcl-2 family of proteins plays a crucial role in the regulation of apoptosis. Thus, in order to understand the molecular or biochemical mechanisms by which actinomycin D induces apoptosis in HL-60 cells, the expression levels of major apoptotic proteins were investigated. The process of apoptosis is regulated by a number of genes and proteins which are targets for anticancer therapy. The Bcl-2 family of proteins is classified as anti-apoptotic and pro-apoptotic and plays a crucial role in the control of apoptosis by regulating mitochondrial membrane permeability (Hofseth *et al*., 2004).

The Bcl-2 (anti-apoptotic) gene encodes a protein that prolongs cell survival by inhibiting factors for the activation of caspases that dismantle cells (Ling *et al*., 2002) or may regulate apoptosis through functional antagonism through the formation of heterodimers with other family members. On the other hand, Bax is a pro-apoptotic member that binds to the anti-apoptotic Bcl-2 protein and thus acts by antagonising Bcl-2's function to abrogate apoptosis. Induction of Bax is also reported to promote the release of cytochrome C from the mitochondria, which eventually leads to apoptosis (Thomas *et al*., 2000).

These anti- and pro-apoptotic proteins are the key regulators of the intrinsic pathway of apoptosis, controlling a point of no return and setting a threshold for the engagement of the death machinery (Matzo and Naval, 2009). Previous reports have shown that the ratio of Bax to Bcl-2 determines in part, the susceptibility of cells to death signals (Cha *et al*., 2004; Paris *et al*., 2007). The expression levels of Bcl-2 and Bax on the treated cells was analysed by

Western blotting. The results presented herein clearly show that Bcl-2 expression was inhibited while Bax expression was significantly increased on treated cells when compared with the untreated cells (Figure 16a); hence the ratio of pro-apoptotic proteins to the anti-apoptotic was altered in favour of apoptosis (Figure 16b). The results of this study show an up-regulation of Bax and the corresponding down regulation of Bcl-2 proteins, which may be one of the critical mechanisms through which actinomycin D induces apoptosis in HL-60 cells. This clearly supports the evidence obtained from the cell toxicity and morphological studies.

5.4 Screening of lectins as biochemical markers of apoptosis

Another approach of this study was to screen several plant lectins for their potential use as biochemical markers of apoptosis, after establishing the early and late stages of apoptosis. Bilyy and Stoika (2003) found that some glycoproteins in the plasma membranes undergo redistributions during apoptosis by screening the cell surface for new biochemical markers of apoptosis that are readily accessible for analysis without damaging the cells. Changes in membrane glycoproteins can be detected histochemically by the use of labelled lectins, which serves as carbohydrate binding proteins (Gabor *et al*., 1998). To detect the cell surface exposure of carbohydrates during induced apoptosis on HL-60 cells, cells in early stages of apoptosis were stained with FITC-labelled lectins. According to our observations the membrane area of HL-60 cells treated with actinomycin D was intensively stained by fluorescein-conjugated *T*. v*ulgaris* compared to all other lectins used in the current study (Figures 17a and b). These results indicate a rather strong interaction between the cell surface and the N-acetylglucosamine binding lectin. The strong interaction of the *T*. *vulgaris* lectin and the cell surface of apoptotic cells might be due to an increased N-acetylglucosamine expression on the exposed cell surface area. Furthermore, this study did, however highlight the sugar specific dissimilarities of the altered cell surface, since none of the lectins used were N-acetylglucosamine specific; hence none or very low cell bound fluorescent was observed.

To estimate the extent of lectin-binding to the apoptotic cells due to protein cell interaction, the HL-60 cells were incubated with dilution series of FITC-labelled *T*. *vulgaris*. As observed in the titration results (Figure 19), the relative cell bound fluorescence intensity increased with an increased lectin concentration, indicating specificity of interaction between the lectin and the altered membrane glycoproteins. This concentration-dependent binding could be attributed to

specificity of interaction to the treated cells. It was also evident that the FITC-labelled *T. vulgaris* not only stained the membranes of apoptotic cells but was also able to stain the interior organelles as well by internalising the stained lectin through the membrane.

In addition, the cells were forming clusters after staining with FITC-labelled *T. vulgaris* lectin. The observed clusters may occur due to by-stander killing due to signal substances released by primary dying cells. This could have been induced by the fluorescent staining on the primary apoptotic cells. Furthermore an increased membrane antigen expression that is also recognised by the *T. vulgaris* lectin could have been triggered by the staining, thus causing cell-cell communication and hence clusters.

The extent of specific binding of the lectin to the HL-60 cells treated with actinomycin D was determined by competitive inhibition of the *T.* v*ulgaris* to the surface of apoptotic cells by the complementary sugar. For the competitive assay, the concentration of *T. vulgaris* was chosen at 500 μg/ml in order to achieve optimum conditions for maximum lectin binding to the cells. The results in the current study showed no fluorescence on treated cells stained with FITC-labelled *T. vulgaris* and pre-incubated with N-acetylglucosamine and BSA (Figure 21) compared with the control cultures which were not preincubated with the inhibitors. Beeckmans *et al*. (2002) stated that binding of the seed lectin from *T.* v*ulgaris* is greatly inhibited by the N-acetylglucosamine. The amount of cell bound labelled lectins decreased, indicating the extent of carbohydrate-mediated binding to the lectins. This could be due to the high inhibitory potency of N-acetylglucosamine resulting in a high decrease in cell bound fluorescence. BSA also seemed to interfere with lectin binding to the treated cells by decreasing the bound fluorescence in a way that is not yet understood.

5.5 Conclusion

In conclusion, this study suggests that large quantities of the *P. angolensis* seed lectin can be easily purified by Sephadex G-75 affinity chromatography; however the supermacroporous affinity matrices can be used as efficient matrices for purifying the lectin from very crude homogenates much quicker with high purity without being blocked. This study also confirms that both the lithium chloride and actinomycin D have an effect on HL-60 tumour cells by inducing cell death, as noticed by reduced cell viability, appearance of the visible nucleosomal

fragments, and morphological changes. In addition the changes in the expression of Bcl-2 family of proteins may also play a prominent role in the actinomycin D-induced apoptotic cell death. Furthermore, these findings provide scientific evidence that supports the research of lithium chloride and actinomycin D as potential anti-cancer agents by altering membrane glycoproteins that are recognised by specific lectins. The results presented further demonstrated, *T. vulgaris* as one of the lectins that recognise altered carbohydrates on the cell surface of apoptotic cells. Thus, *T. vulgaris* can be used as a potential marker to detect apoptosis by binding specifically to the carbohydrate component of the glycoprotein molecule expressed on the plasma membrane during apoptosis. Further investigation into the effect of the *T. vulgaris* on actinomycin-D treated cells is required. These follow-up experiments will result in exploring ways to enriching the apoptotic cells by separating them from the non-apoptotic cells using the lectin-activated supermacroporous cryogels.

CHAPTER 6

REFERENCES

Adams, J. M. and Cory, S., 1998. The Bcl-2 protein family. Arbiters of cell survival. *Sci.*, **281**, 1322-1326

Ahlqvist, J., Kumar, A., Sundstrom, H., Ledunh, E., Horntein, E.G., Enfors, S., and Mattiasson, B., 2006. Affinity binding of inclusion bodies on supermacroporous monolithic cryogels using labeling with specific antibodies. *J. Biotechnol.*, **122**, 216- 225

Appelt, U., Sheriff, A., Gaipl, U.S., Kalden, J.R., Voll, R.E., and Herrmann, M., 2005. Viable, Apoptotic and Necrotic monocytes expose phoaphstidyl serine: Cooperative binding of the ligand Annexin-V to dying but not viable cells and implications for PS-dependent clearance. *Cell Death Differ.*, **12**, 194-196.

Amadeo, G.I., Moreira, R., Lima, R., Teixera, D., Kratje, R. and Cheverrigaray, M., 2005. Screening of lectins from South American plants used as affinity ligands to purify rhEPO. *Braz. J. Chem. Eng.*, **20 (**1), 662-670

Amano, J., Kobayashi, K., and Oshima, M., 2001. Comparative Study of Glycosyltransferase activities in Caco-2 cells before and after Enterocytic Differentiation Using Lectin-Affinity High-Performance Liquid Chromatography. *J. Biochem. Biophysic.*, **395**(2), 191- 198

Aoki, I.J., Kawano, T., Oinuma, Y., Haraguchi, T., and Suganuma, T., 1993. Human colorectal carcinoma specific glycoconjugates detected by pockeweed mitogen lectin. *Histochemistry*, **41**, 1321-1330.

Arvidsson, P., 2002. Polymer displacement and supermacroporous cryogels- Two new tools in chromatographic separation of biomolecules and bioparticles. Doctoral Dissertation, Department of biochemistry, Lund University, Sweden. pp 3-36

Arvidsson, P., Plieva, F.M., Savina, I.N., Lozinsky, V.I., Fexby, S., Bülow, L., Yu. Galaev, I. and Mattiasson, B., 2002. Chromatography of microbial cells using continuous supermacroporous affinity and ion- exchange columns. *J. Chromatogr. A*, **977 (**1), 27-38

Arvidsson, P., Plieva, F.M., Lozinsky, V.I., Yu. Galaev, I. and Mattiasson, B., 2003. Direct chromatographic capture of enzyme from rude homogenate using immobilized metal affinity chromatography on a continuous supermacroporous adsorbent. *J. Chromatogr.* A, **986**, 275-290

Babac, C., Yavuz, H., Galaev I.Y., Piskin, E., and Denizli, A., 2006. Binding of antibodies to Concavalin A modified monolithic cryogel. Reactive and functional polymers, *J. Chromatogr.,* **66**, 1263-1271

Bakalova, R. and Ohba, H., 2002. Purification of normal lymphocytes from leukemic T-cells by Lectin-affinity adsorbents: Correlation with Lectin-cell binding. *Cancer. Lett.,* **192 (**1), 59-65

Barre, A., Bourne, Y., Van Damme, J.M., Peumans, W.J., and Rougé, P., 2001. Mannose-binding plant lectins: Different structural scaffolds for a common sugar-recognition process. *J. Biochemica.*, **83**, 645−651

Becker, R. W. and Tyobeka, E. M., 1996. Lithium effects on proliferation, differentiation and toxicity in cell cultures. *Weidner Publ Group*, Cheshire, Conneticut, Pg 19-37

Beeckmans, S., De Greve, H., Blanca, D.E., Loris, R., Read, J.S., Ncube, I., and Van Driessche, E., 2002. A comparative study on seed lectins from *Pterocarpus* species. *J. Biochem.*, **268**, 103-109

Bilyy, R. O., Antonyuk, V. O., and Stoika, R. S., 2004. Cytochemical study of the role of alpha-D-mannose and beta-D-galactose containing glycoproteins in apoptosis. *J. Mol. Histol.*, **35**, 829-838

Bilyy, R.O., and Stoika, R.S. 2003. Lectinocytochemical detection of apoptotic murine leukemia L1210 cells. *Cytometry*, **56A**, 89-95.

Braun, R., Teute, H., Kirchner, H., and Munk, K., 2000. Rapid separation of T cells with monoclonal antibodies and affinity chromatography. *J. Chromatogr.* A, **1045**, 93-98

Cha, Y-Y., Lee, E-O., Lee, H-J., Park, Y-D., Ko, S-G., Kim, D-H., Kim, H-M., Kang, I-C. and Kim, S-H., 2004. Methylene chloride fractionation of *Scutellaria barbata* induces apoptosis in human U937 leukemia cells via the mitochondrial signaling pathway. *Clinica. Chemica. Acta,* **348**, 41-48

Chang, H.Y, and Yang, X., 2000. Proteases for cell suicide: functions and regulations of caspases. *Microb. Mol. Biol. Review* **64** (**4**), 821-846

Cheng, A-N., Huang, T-C., Lai, C-S., and Pan, H., 2005. .Induction of apoptosis by Luteolin through cleavage of BCL-2 family in human leukemia HL-60 cells. *European J. Pharmacol.*, **509**, 1-10

Cho, S.G., and Choi, E.J., 2002. Apoptotic signaling pathways: Caspases and stress-activated protein kinases. *J. Biochem. Mol. Biol.*, **35**, 24-27

Cummings B. S., Schenellmann R. G., 2002. Cisplatin induced renal cell apoptosis: caspase-3 dependent and independent pathways. *J. Pharmacol. Experim. Therap.*, **302**, 8-17

Dabelsteen, E., Mandel, U., Clausen, H.,1991. Cell surface carbohydrates as markers of differentiation in human oral epithelium. *Crit. Rev. Oral Biol Med.*, **4,** 493-507

Dainiak, M. B., Plieva, F. M., Yu Galaev, I., Hatti-Kaul, R. and Mattiasson, B., 2005. Cell Chromatography: Separation of different microbial cells using IMAC supermacroporous columns. *Biotechnol. Prog.*, **283**, 185-194

Dainiak, M.B., Kumar, A., Plieva, F.M., Yu. Galaev, I. and Mattiasson, B., 2004. Integrated isolation of antibody fragments from microbial cell culture fluids using supermacroporous cryogels. *J. Chromatogr.* A, **1045**, 109-112

Davidson, E and Stewart, D., 2004. Novel mannose binding lectins from agricultural crops. *J. Ind Crops and Products*, **19**, 137–146

Echemendia-Blanco, D., Van Driessche, E., Ncube, I., Read, J. S. and Beeckmans, S., 2009. Stability, subunit interactions and carbohydrate-binding of the seed lectin from *Pterocarpus angolensis*. *Prot. Pept Letter*, **16**, 1120-1134

Fujimura, M., Morita-Fujimura, Y., Murakami, K., Kawase, M., and Chan, P.H., 2002. Cytosolic redistribution of cytochrome C after transiet focal cerebral inschemia in rats. *J. Cereb. Blood Flow Met.*, **18**, 1239-1247.

Gabius, H. J., Gabius, S., Zemlyanukhina, T.V., Bovin, N.V., Brinck, U., Danguy, A., Josshi, S.S., Kayser, K., Schottelius, J., Sinowatz, F., Vidal-Vanacocha H., and Zanetta, J., 1993. Reverse Lectin Histochemistry: design and application of glycoligands for detection of cell and tissue lectins. *Histol. Histopath., 8,* 386-383

Gabor, F., Bogner, E., Weissenboeck, A., and Wirth M.., 2004. The lectin–cell interaction and its implications to intestinal lectin-mediated drug delivery. *Adv. Drug Delivery Reviews*, **56,** 459– 480

Gabor, F., Stangl, M. and Wirth, M., 1998. Lectin mediated bioadhesion: Binding characteristics of plant lectins on the erythrocyte-like cell lines CaCo-2, HT-29 and HCT-8. *J. Contr. Release.*, **55**, 131-142

Goldstein, J and Porets, R.D., 1986. Isolation, Physiochemical, characterization, and carbohydrate-binding specificity of lectins. *Glycop.*,**8**, 76-80

Goldstein, I.J., Winter, H.C., and Poretz, R.D., 1997. Plant lectins: tools for the studying of complex carbohydrates, In: Montreuil, J., Vliegenthart, J.F.G & Schater, H., Editors, *Glycop.*, **16**, 403-474

Goossens, J. F., Henichart, J. P., Dassonneville, L. and Facompre, C.B., 2000. Relation between intracellular acidification and camptothecin induced apoptosis in leukemia cells. *European J. Pharmacol Sci.*, **10**, 125-131

Graz. F. D., 2004. Description and Oncology of *Pterocarpus angolensis* in Namibia. *Dinteria No* **29**, 27- 39

Hanora, A., Savina, I., Plieva, F.M., Izumrudov, A.V., Mattiasson., B., and Galaev, I.Y., 2006. Direct capture of plasmid DNA from non-clarified bacterial Lysates using polycation-crafted monoliths, *J. Biotechnol.*, **123**, 343-355

Hedemann, M.S., Hojsgaard, S., and Jensen, B.B., 2007. Lectin histochemical characterization of the porcine small intestine around weaning. *Researc. Vet. Sci.*, **82**, 257–262

Hengartner M. O. 2000. The biochemistry of apoptosis. *Nature*, **12,** 770-776

Heyder, P., Gaipl, U.S., Beyer, T.D., Voll, R.E., Kern, P.M., and Stach, C., 2003. Early detection of apoptosis by staining acid-treated apoptotic cells with FITC-labelled lectins from Narcissus pseudonarcissus. *Cytometry*, **55A**, 882-893

Hirabayashi, J., 15-12-1997. Introduction to "Lectins". Accessed on 06-07-2006 from http://www.glycoforum.gr.jp/science/word/lectin/LEA00E.html

Hofseth, L. J., Hussain, S. P. and Harris, C. C., 2004. p53; 25 yrs after its discovery. *Trends Pharmacol. Sci*, **25** (4) 177-181

Hossain, M.A., Rafiqul Islam, S.M., and Abscar, N., 2004. Purification and Characterization of Lectins from mulberry seeds (morus *alba L.*) *Pakistan J. Biol. Sciences,* **10,** 1803-1813

Hussain, S. P., Hofseth, L. J., and Harris, C. C., 2003. Radical causes of Cancer. *Nature Reviews Cancer*, **3**, 276- 285

Iams, K.P., 19-07-1999. Present research interest. Accessed 08- 08-2006 from http://216.109.125.130/search/cache?p=Dr.Keith=Paul%2Czimbabwe&prssweb=Search&ei=UTF&&fl=0&u=www.uz.ac.zw/science/biochemistry/staff/iams.html&w=dr+keith+paul+zimbabwe&d=cq

Jin, U.-H., Lee, D.-Y., Kim, D.-S., Lee, I.-S., and Kim, C.-H., 2006. Induction of mitochondrial-mediated apoptosis by methanol fraction of *Ulmus davidiana* planch (Ulmaceae) in U87 glioblastoma cells. *Environ. Toxic. Pharmacol.*, **22**,136-141

Kamakoff, W., 28-01-2007. Apoptosis. Accessed 12-11-2007 from www.microbiologybytes.com/.../pics/Apoptosis.gif

Khan, F., Khan, R., Sherwani, a., Mohmood, S., Asfer, A., 2002. *Med. Sci, Monitor.*, **8**, 293-300

Kottgen, E., Kage, A., Hell, B., and Tauber, R., 1993. Glycoprotein-lectin-immunosorbent assay. *J. Immunol.*, **13**, 41-46

Kumar, A., Bansal, V., Andersson, J., Roychoudhury, P. K., and Mattiason, B., 2006. Supermacroporous cryogel matrix for integrated protein isolation. Immobilized metal affinity chromatographic purification of urokinase from cell culture broth of a human kidney cell line. *J. Chromatogr.* A, **1103**, 35-42

Kumar, A., Rodriquez-Caballero, A., Plieva, F.M., Yu Galaev, I., Nandakumar, K.S., Kamihira, M., Holmdahl, R., and Orfao, A., 2005. Affinity binding of cells to cryogel adsorbents with immobilized specific ligands: effect of ligand coupling and matrix architecture. *J .Mol. Recognit.*, **18**, 84-93

Kumar, A., Plieva, F.M., Yu Galaev, I., and Mattiasson B., 2004. Affinity Fractionation of Lymphocytes using a monolithic cryogel. *J. Immunol. Methods.*, **283**, 185-194

Kumar, A., Plieva, F.M., Yu Galaev, I., and Mattiasson B., 2003. Affinity Fractionation of Lymphocytes using a monolithic cryogel. *J. Immunol. Methods.*, **283**, 185-194

Laemmli, U.K, 1970.Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature*, **270**, 680-685.

Lawen, A., 2003. Apoptosis- an introduction. *Bioassays*, **25**, 888-896

Lewandrowski, U., Moebins J, Walter, U., and Sickmann, A., 2005. Elucidation of N-Glycosylation sites on human platelet Proteins: A glyco-proteomic approach. *Mol. Cell Proteomics*, **220,** 103- 109

Ling, Y. H., Liebes, L., Ng, B., Buckley, M., Elliot, P. J., Adams, J., Jiang, J. D., Muggia, F. M. and Perez-Soler, R., 2002. PS-341, A novel proteasome inhibitor, induces Bcl-2 phosphorylation and cleavage in association with G_2-M phase arrest and apoptosis. *Mol. Cancer. Ther.*, **1**, 841-849

Loris, R., Imberty, A., Beeckmans, S., Van Driessche, E., Read, J.S., Bouckaert, J., De Greve, H., and Wyns, L., 2003. Crystal Structure of *Pterocarpus angolensis* Lectin in complex with glucose, sucrose, and turanose. *J. Biochem.*, **278**(128), 16972-16303

Loris, R., Boukaert, J., Buts, J., de Greve, H., and Wyns, L., 2002. Structural basis of protein carbohydrate interaction by the *Pterocarpus angolensis* seed Lectin. *Immunology*, **109**(1), 93-101.

Loris, R., 2002. Principle of structure of animal and plant Lectins. *Biochem. Biophysic. Acta.*, **1572**(2), 198-208.

Lozinsky, V. I., Galaev, I. Y., Plieva, F.M., Savina, I. M., Jungvid, H., Mattiasson, B., 2003. Polymeric cryogels as promising materials of biotechnological interest. *Trends in Biotechnol.*, **21**, 445-451

Lu, Y, and Chaney, W., 1993. Induction of N-acetylglucosamyltransferase by elevated expression of activated or pro-ras oncogenes, *Mol. Cell. Biochem.*, **122**, 82-89

Lucas, K.C., 2005. BIOC 331, Biochemical Techniques. University of Limpopo Press, Sovenga, South Africa. 84-87

Madiehe, A.M., Mampuru, L.J. and Tyobeka, E.M., 1995. Induction of apoptosis in HL-60 cells by lithium. *Biochem. Biophysic.*, **209** (2), 768-774

Matsebatlela, T.M., Mogodiri, R.K., Hart, D.A., Gallicchio, V. S. and Becker, R.W., 2000. Resistance to lithium induced apoptosis in a lithium tolerant clone of HL-60 promyelocytes. *J. Trace. Micro.*, **18**, 163-170

Marzo, I. and Naval, J., 2008**.** Bcl-2 family members as molecular targets in cancer therapy. *Biochem. Pharmacol.*, **76** (8), 939-946

Mbazima, V. G., Mokgotho, M. P., February, F., Rees, D. J. G. and Mampuru, L. J., 2008. Alteration of Bax-to-Bcl-2 ratio modulates the anticancer activity of methanolic extract of *Commelina bengalensis* (Commelinaceae) in Jurkat T cells. *Afri. J. Biotechnol.*, **7**(20), 3569-3576

Min-Liang, K., Tse-sing, H., and Jen-kun, L., 1996. Curcumin, an antioxidant and antitumour promoter, induces apoptosis in human leukemia cells. 1996. *Biochemic. Biophysic. Acta.*, **1317,** 95-100

Mody, R., Joshi, S.,and Chaney, W., 1995. Use of lectins as diagnostic and therapeutic tools for cancer, *J. Pharmacol.Toxic. Methods.*, **33**, 1-10

Monzo, A., Bonn, G. K., Guttman, A., 2007. Lectins-immobilization strategies for affinity purification and separation of glycoconjugates. *Trends. Analyt. Chemistry.*, Vol 26, **5**. 423-432

Nandakumar, M.P, and Mattiasson, B., 1999. Purification of horse radish peroxidase by metal affinity chromatography in an expanded bed system. *Biotechnol. Techniq.*, **13,** 671- 675

Ndamba, J., Nyazema, N., Makaza, N., Anderson, C. and Kaondera, K.C., 1994. Traditional herbal remedies used for the treatment of urinary schistosomiasis in Zimbabwe. *J. Ethnopharmacol.*, **42**, 125-132

Oliveira, S. R. M., Nascimento, A. E., Lima, M. E. P., Leite, F. M. and Benevides, M. B., 2002. Purification and characterization of lectins from red marine alga: *Pterocladiella capillacea*. *Rev. Bras. Bot.*, **25**, 397-403

Paris, C., Bertoglio, J. and Breard, J., 2007. Lysosomal and mitochondrial pathways in milterfosine induced apoptosis in U937 cells. *Apoptosis*, **12**, 1257-1267

Plieva, F.M., Savina, I.N., Deraz, S., Yu. Galaev, I. and Mattiasson, B., 2004. Characterization of Supermacroporous monolithic polyacrylamide based matrices designed for Chromatography of bioparticles. *J. Chromatogr. B,* **807**,*1*29-137

Porras, F., Urrea, F., Ortiz, B., Martinez-Cairo, S., Bonquelet, S., Martinez, G., Lascurain, R., and Zenteno, E., 2005. Isolation of receptor for *Amaranthus leucocarpus* Lectin from human T-lymphocytes. *Biochem. Biophys Acta*, **1726**(1-2), 155-162

Putnam, D.D., Navasivayam, V., and Burns, M. A., 2003. Cell Affinity separations using magnetically stabilized fluidized beds; Erythrocyte subpopulation fractionation utilizing a Lectin-Magnetide Support. *Biotechnol. Bioeng.*, **81**, 650-665

Ravindranath, M. H., Bauer, P.M., Cornillez-Ty, C., Garcia, J., Morton, O.L., 2006. Quantitation of the density of cell surface carbohydrates antigens of cancer cells with a sensitive cell-suspension ELISA. *J. Immunol. Meth.*, **197**,51-67

Reddy, L., Odhav, B. and Bhoola, K.D., 2003. Natural products for cancer prevention: a global perspective. *Pharmacol. Therapeutics,* **99**: 1-13.

Reisner. Y, and Gan, J., 1983. Differential agglutination by soybean agglutinin of human leukemia and neuroblastoma cell lines: potential application to autologous bone marrow transplantation. *Proc. Natl. Acad. Sci., USA*, **80**, 6657–6661

Reed. J.C., 1994. BCL-2 and the regulation of programmed cell death. *J. Cell.Biology,* **124**, 1-6.

Rudiger, H., and Gabius, H.J., 2001. *J. Glycoconj.*, **18**, 569-613

Savina, I.N., Yu. Galaev, I. and Mattiasson, B., 2005. Anion-Exchange Supermacroporous Monolithic Polyacrylamide Matrices with grafted polymer brushes of N,N-dimethylaminoethyl-methacrylate. *J. Chromatogr.* A, **1092**, 199-205

Satish, P.R, and Surolia, A., 2001. Exploiting lectin affinity chromatography in clinical diagnosis, *J. Biochem. Biophys. Methods*, **49**, 625-640

Sengbusch, P.V., 07-31-2003. Lectins. Accessed on 08-11-2005 from http://www.biologie.uni-hamburg.de/b-online/e17/17h.htm

Sharon, N. and Lis, H., 1993. Carbohydrates in cell recognition. *Sci. Amer.*, **268**, 82-92

Sharon, N. and Lis, H., 1990. Lectins 2nd ed. Kluwer Academic Publ, USA, 261-311

Smith, P.K., Krohn, R. I., Hermanson, G. T., Mallia, A.K., Garhter, F.H., Provenzano, M. D., Figimito, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C., 1985. Measurements of proteins using bicinchoninic acid. *Anal. Biochem.*, **150**, 76-85

Srinivas, V.R., Reddy, G. B., Ahmad, N., Swaminathan, C. P., and Surolia, N.M.A., 2001. Legume lectin family, the natural mutants of the quaternary state, provide insights into the relationship between protein stability and oligomerization. *Biochem. Biophysic. Acta.*, **1527**, 102-111

Taha, T. A., Osta, W., Kozhaya, L., Bielawski, J., Johnson, K. R., Gillander, W. E., Dbaiba, G. S., Hannun, Y. A. and Obeid, L. M., 2004. Down-regulation of sphingosine kinase-1 by DNA damage. *J. Biol. Chem.*, **279** (19), 20546-20554

Thomas, A., Giesler, T. and White, E., 2000. p53 mediates Bcl-2 phosphorylation and apoptosis via activation of the Cdc42/JNK1 pathway. *Oncogene*, **19**, 5259-5269

Thompson, C.B., 1995. Apoptosis in the pathogenesis and treatment of disease. *Sci.*, **267**, 1456-1462

Van Engeland, M., Ramaekers, F.C.S., Schutte, B., and Reutelingperger, P.M., 1996. A novel asasay to measure loss of plasma membrane asymmetry during apoptosis of adherent cell in culture. *Cytometry*, **24**, 131-139

Vermes, I., Haneen, C., Steffens-Nakken, H., Reutelingsperger, C., 1995. A novel assay for apoptosis: flof cytometric detection of phosphotidyl serine expression on early apoptotic cells using Flourescein labeled Annexin-V. *J. Immunol, Methods*, **184**, 39-51

Vornholt, W., Hartmann, M., Keusgen, M., 2007. SPR-studies on carbohydrate lectin interactions as useful tool for screening on lectin sources. *Biosens. Bioelectron.*, **22,** 2983-2988

Weisenthal, L. M., Marsden, J. A., Dill, P. L. and Macaluso, C. K., 1983. A novel dye exclusion method for testing *in vitro* chemosensitivity of human tumours. *Cancer Research*, **43**, 749-757

Wyllie, A.H., 1987. Apoptosis: cell death in tissue regulation. *J. Pathol.*, **153**: 313-336.

Yi J-M., Kim M-S., Lee E-H., Wi D-H., Lee J-K., Cho K-H., Hong S-H. and Kim H-M., 2003. Induction of apoptosis by *Paljin-Hangahmdan* on human leukemia cells. *J. Ethnopharmacol.*, **88**:79-83

Zhang, X., Moilanen, E., and Kankaaranta, H., 2000. Enhancement of human eosinophil apoptosis by fluticasone propionate, budenoside and beclomethasome. *Eur.J. Pharmacol.*, **406**, 325-332

Zimmermann, K.C., Bonzon, C. and Green, D.R., 2001. The machinery of programmed cell death. *Pharmacol. Therapeut.*, **92**: 57-70.