3. Results

Blood group specificity and sugar inhibition:

Crude extract of *Momordica Balsamina* seeds contains a hemagglutinating activity that agglutinated some animal blood cells and all human blood cells with more activity towards the O blood type as shown in Table 3.1.

The hemagglutinating activity was inhibited by D-galactose and lactose, with lactose showing strong effect, as shown in Table 3.2**.**

Momordica Balsamina seeds lectin purification

The purification procedures of this lectin from seed extract of *Momordica balsamina* are summarized in Table 3.3. Standard curves used for estimation of protein and carbohydrates shown in Figures 3.1, 3.2, 3.3.

Native and subunit structure of *Momordica Balsamina* seeds lectin

The Molecular weight of the intact lectin was examined by gel filtration on sephadex G-100; calibrated with protein standards as shown in figure 3.4 and table 3.4, the result indicated that the native molecular weight is 81kDa as shown in Figure 3.5. The lectin was further analyzed by SDS-PAGE for revealing its subunit molecular mass in the presence of 2- mercaptoethanol. As depicted in Figure 3.6; lectin yielded a single band with molecular mass of 30kDa.

Effect of pH on *Momordica Balsamina* seeds lectin

Effect of pH on lectin studied in pH range from 2-13, and the lectin was stable over a wide range of pH values as shown in figure 3.7.

Effect of thermal stability on *Momordica Balsamina* seeds lectin

The effect of temperature on lectin studied in range of temperature (20 – 90 ^{o}C) and the lectin was stable over a wide range of temperature as shown in figures 3.8.

Effect of urea as denaturing agent on *Momordica Balsamina* seeds lectin

Purified lectin when incubated with different concentrations of urea showed slightly decreased activity up to 3M of urea concentration as shown in Figure 3.9.

N-terminal sequencing of lectin peptides

Momordica Balsamina seeds lectin was purified by reversed-phase chromatography (Figure 3.10). The eluted peaks 1 and 2 were sequenced by Edman degradation (Figure 3.11). Figure 3.12; showed the sequence alignment of the initial N-terminal of the peak 1 and MCL1, after searching for identity by using BLAST.

Evaluation of cytotoxicity using the MTT Assay

After evaluation of Cytotoxicity of the purified lectin using the MTT Assay inAGS (Human Gastric Adenocarcinoma), MKN45 (Human Gastric Cancer), U87-MG (Human Glioblastoma) and ECV-304 (Human Urinary Bladder Carcinoma) cell lines (as described in methodology 2.2.12.), the results are shown in figures 3.13, 3.14.

Table 3.1: Hemagglutinating activity of *Momordica Balsamina* seed lectin against different blood cells.

Erythrocytes type	Trypsin untreated (Specific activity)	Trypsin treated (Specific activity)
Human		
1) A	16	32
2) B	8	8
3) AB	4	130
4) O	16	130
Animal		
1) Mice	0.08	0.02
2) Donkey	NAD	NAD
3) Cow	0.8	NAD
4) Goat	NAD	NAD

NAD: No agglutination detected

Table 3.2: Carbohydrate Inhibition of agglutination by Momordica Balsamina seed lectin.

	MIC (mM)
Arabinose	100
Glucose	NI
Galactose	50
Mannose	NI
Maltose	NI
Sucrose	NI
Lactose	25

MIC: minimum inhibitory concentration, NI: sugar not inhibitory up to a concentration of 200mM.

Table 3.3: Purification of *Momordica Balsamina* seeds lectin

Stage of purification	volume (ml)	Protein conc. (mg/ml)	Total Protein (mg)	Total CHO (mg)	Lectin Activity (U/ml)	Total Activity (U/ml)	Specific Activity (U/mg)	Fold purification	% Yield
Saline extract	240	27	6480	26	216	51840	8	1	100
Affinity Chromat-ography	15	1.2	18	0	2048	30720	1707	213	59

- Saline extract started from 100g dry floor of *Momordica Balsamina* seeds.
- Human blood group O erythrocytes were used for the assay.
- Total activity (Hemagglutination unit) is defined as the lectin activity multiplied by the total volume.
- Specific activity is defined as the hemagglutination unit divided by the total protein concentration.
- Fold purification is defined as the specific activity of affinity chromatography fraction divided by the specific activity of the saline extract.
- % Yield is defined as the total activity of affinity chromatography fraction divided by the total activity of the saline extract multiplied by 100.

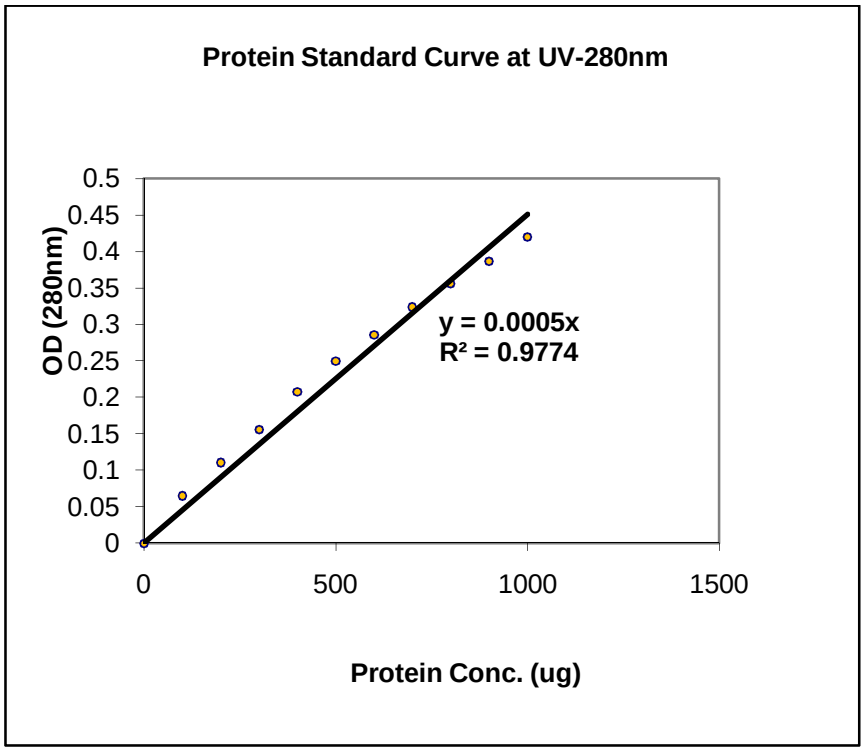

Figure 3.1: protein standard curve at 280nm for protein estimation

Working standard of 100 micrograms/ml was prepared from BSA 1mg/ml standard solution. Serial dilutions of working solution were performed and OD was measured at 280nm.

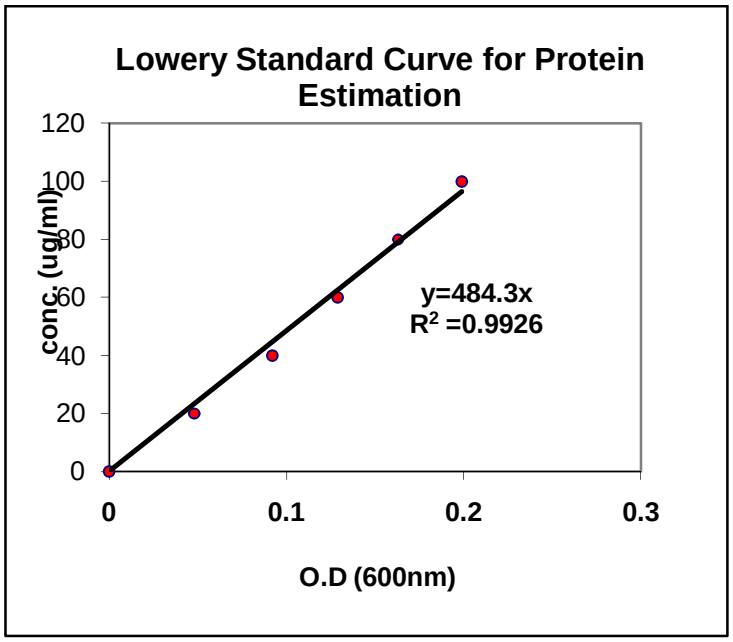

Figure 3.2: Lowery Standard Curve at 600nm for protein estimation

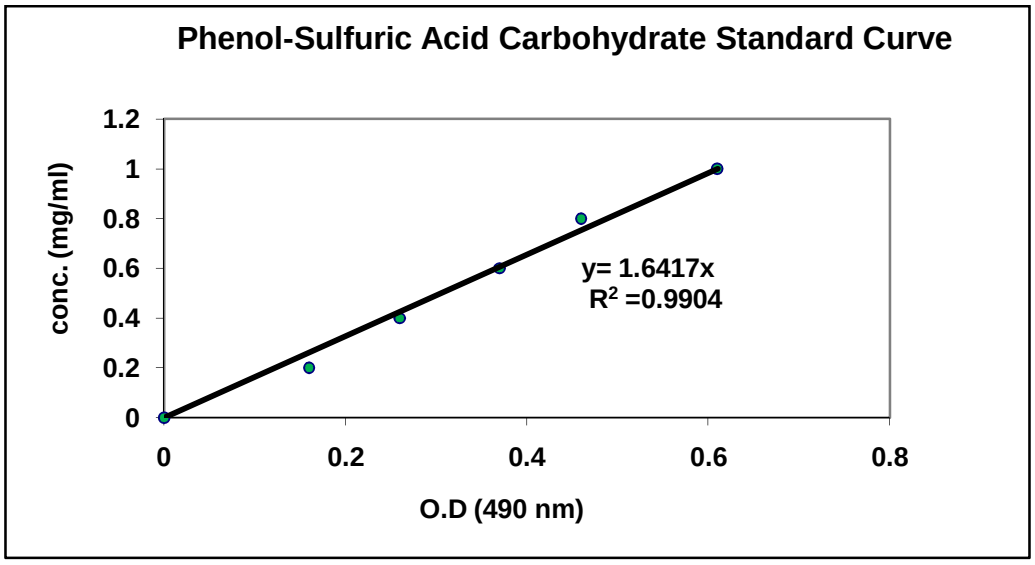

Figure 3.3: Carbohydrate Phenol-Sulfuric acid Standard Curve for carbohydrates estimation.

1 mg/ ml of 100 mM glucose was used as a standard solution, serial dilutions were done and the phenol-sulphuric acid reaction was read at 490 nm.

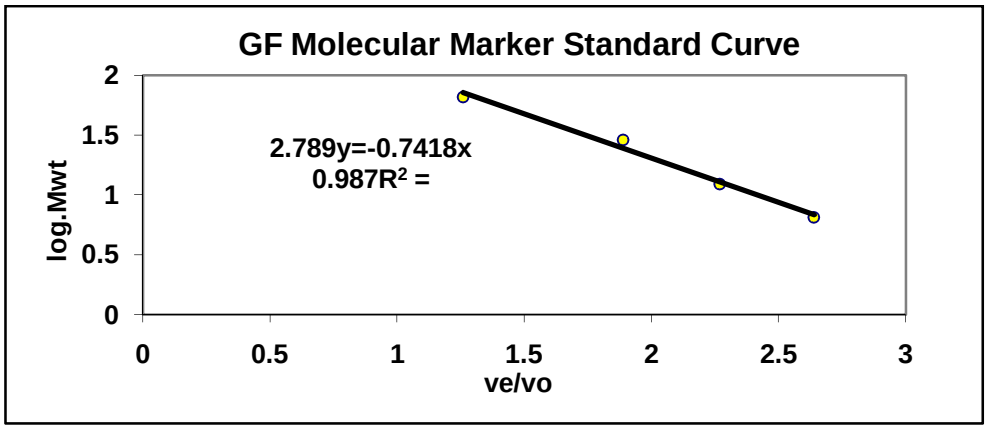

Figure 3.4: Gel Filtration Molecular weight Standard Curve (Vo= 45ml)

The logarithms of molecular marker weights were plotted against the ratio of elution volume of the highest peak at 280 nm and the void volume (the volume at which the highest peak of blue dextran was observed.

Marker	Conc Loaded Mg/ml	Mr.Wt kDa	Log(M.Wt)	V_e ml	V_e/V_o
Albumin	6	66	1.82	57	1.26
Carbonic Anhydrase	5	29	1.46	85	1.89
Cytochrome C	4	12.4	1.09	102	2.27
Aprotinin	4	6.5	0.81	119	2.64

Table 3.4: Gel Filtration Molecular weight Standards (Vo= 45ml)

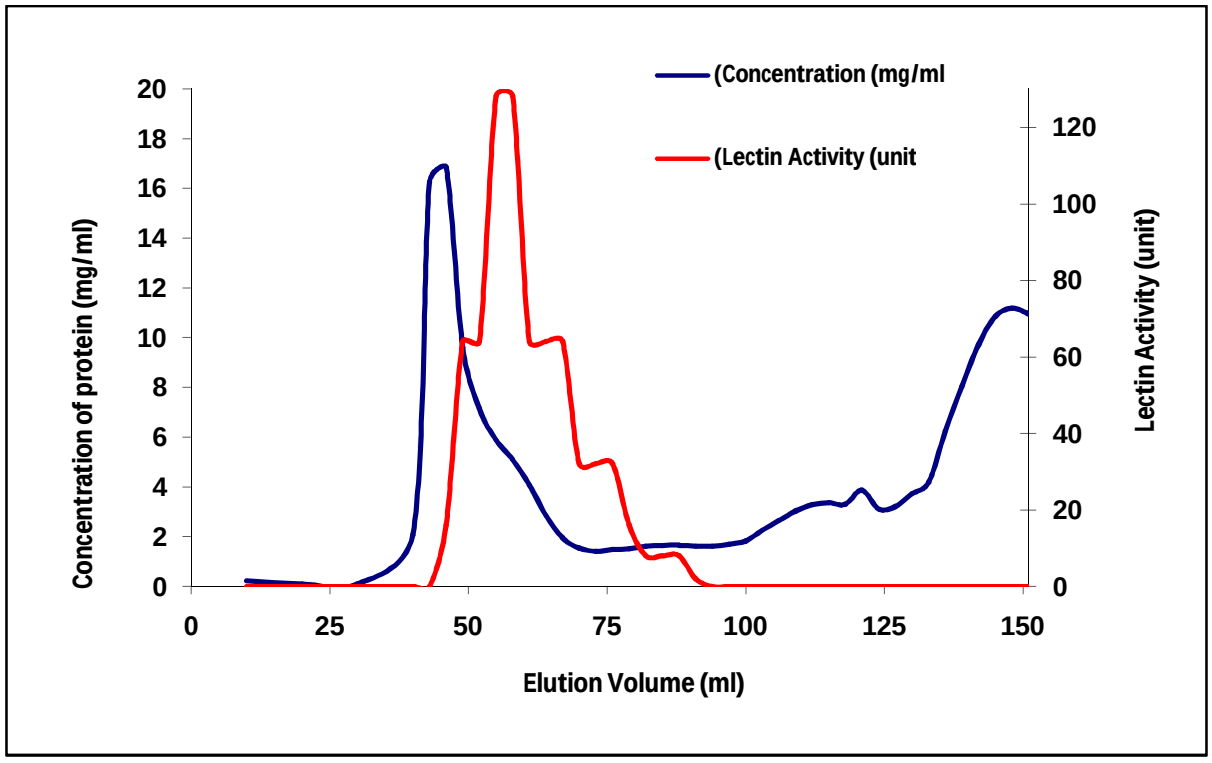

Figure 3.5: Profile of gel filtration of crude extract along with lectin activity curve.

80mg of the crude extract were loaded to the column of 64cm height and 1.5ml/min flow rate, 16 eluted fractions reported positive to presence of lectin after hemagglutination assay with 4% O+ RBCs. The blue line represents the protein profile of the crude extract after GF; the red line represents the activity unit of the lectin. After performing the activity test for lectin the molecular weight is 81kDa.

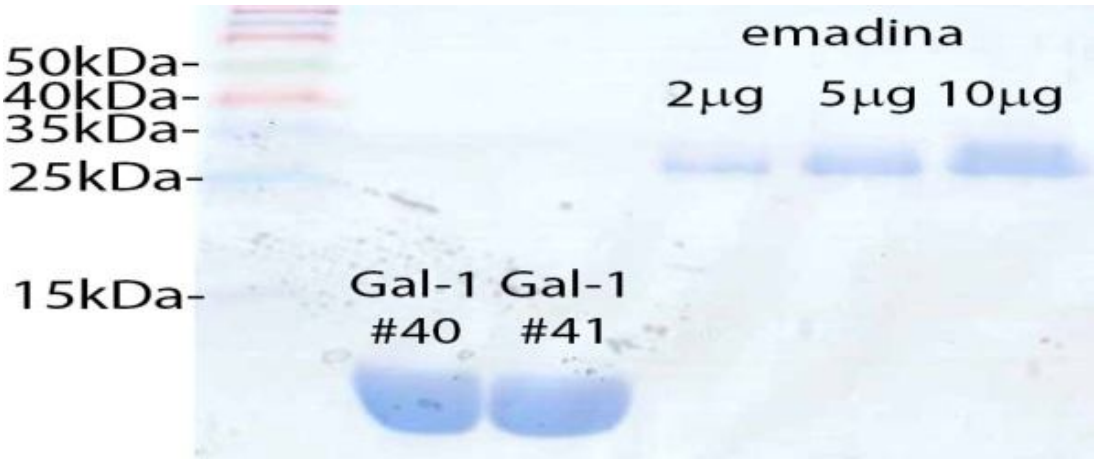

Figure 3.6: SDS-PAGE of the purified lectin denoted emadina

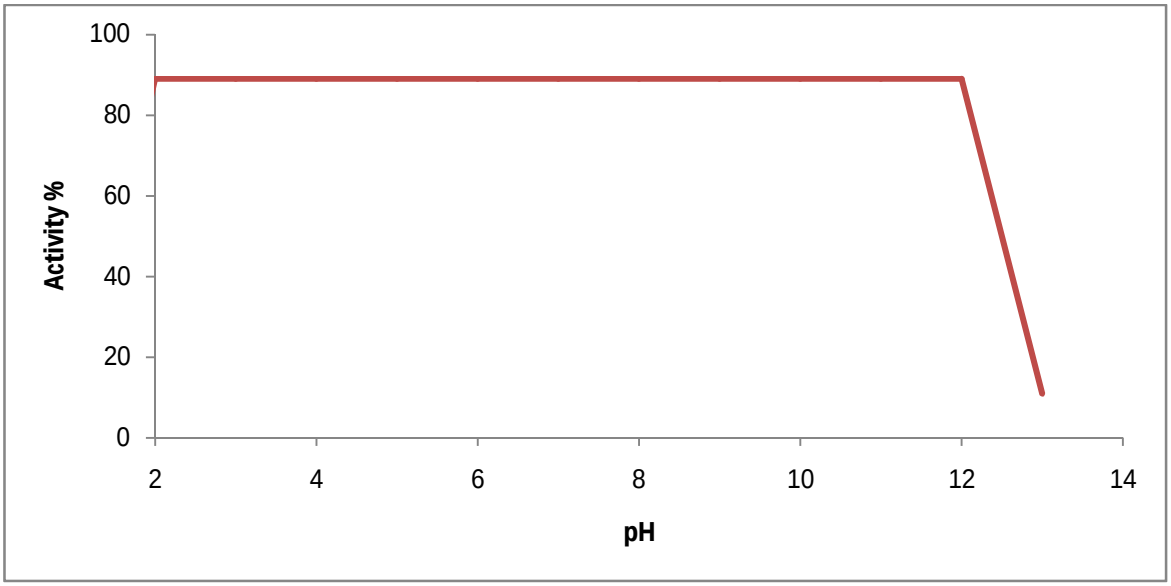

Figure 3.7: Effect of pH on the purified lectin

30ug of Lectin was incubated an hour at varing buffers ranging (2-13) at room temp. Lectin samples were neutralized either by 1% Hcl or 1% NaoH.

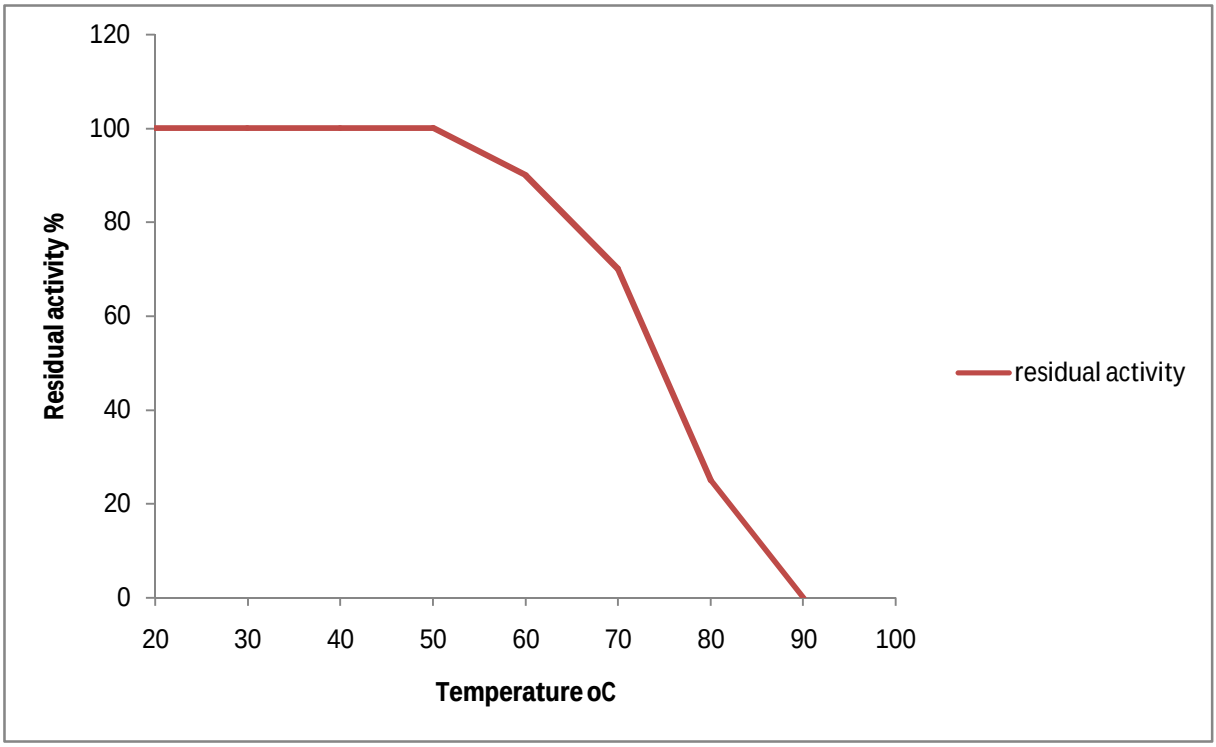

Figure 3.8: Effect of thermal stability on purified lectin.

30ug of lectin was incubated in different temp. interval starting from 20^{o}C to 90^{o}C for half an hour, then the residual activity was calculated. The maximum stability appears in 30^{o}C.

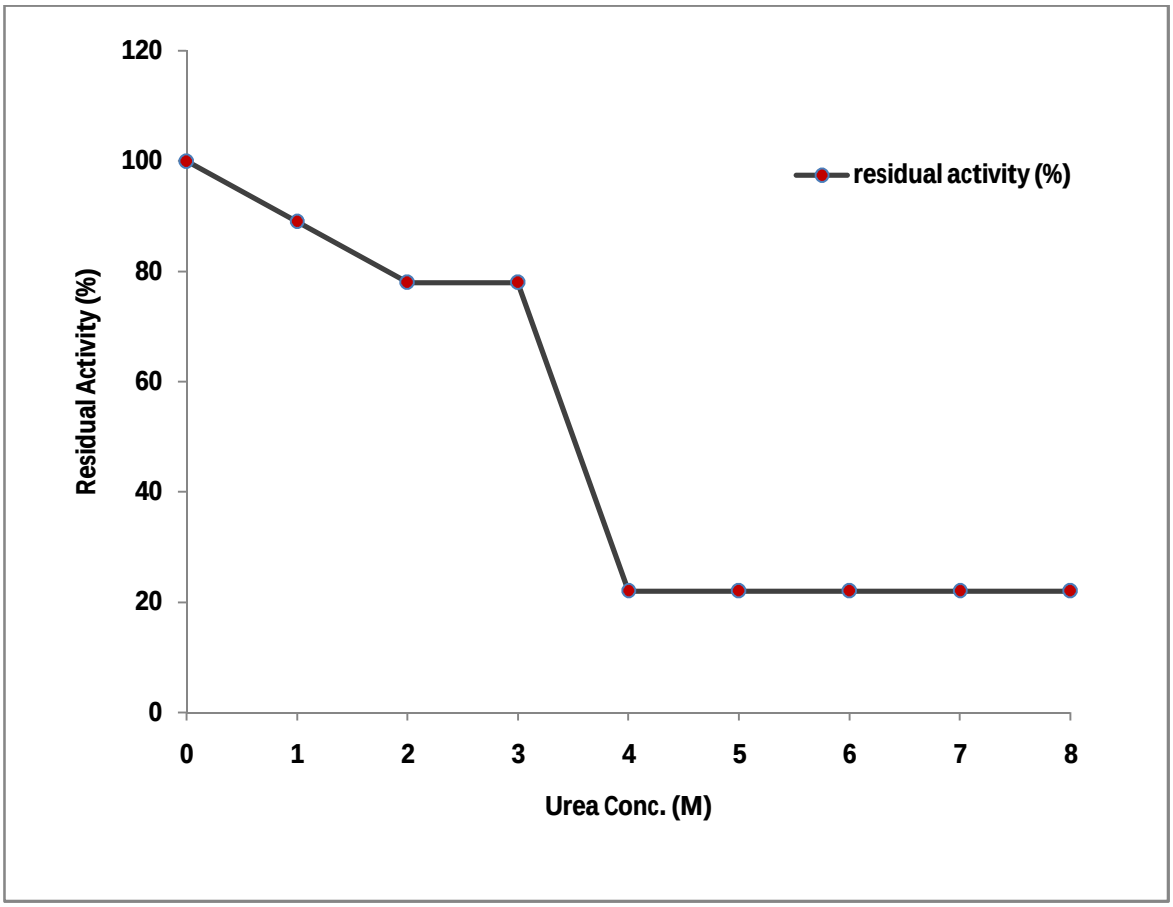

Figure 3.9: Effect of urea on the activity of purified lectin

30ug of lectin was incubated with urea as denaturing agent with different conc. (1-8M) in room temperature for 2 hours, and then the residual activity was calculated. The maximum stability appears in 1M.

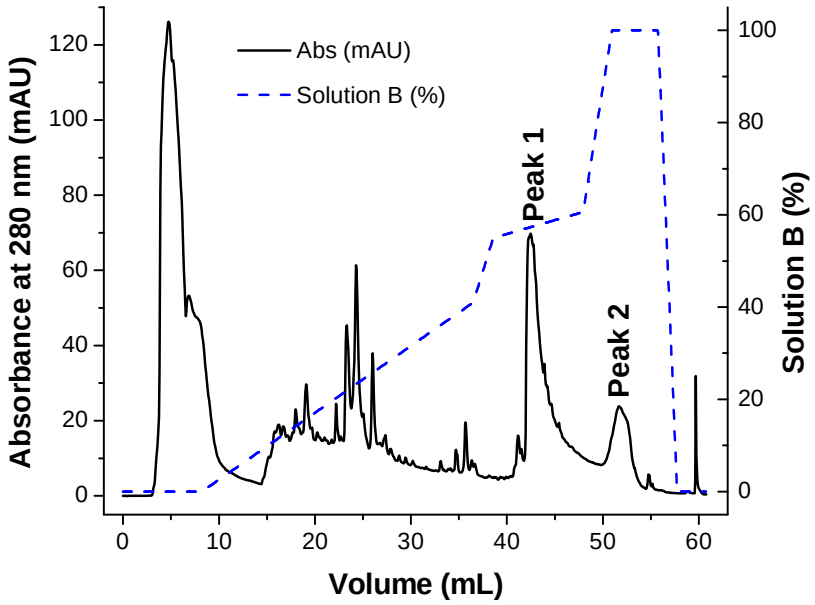

Figure 3.10: Elution profile of lectin using Reversed-Phase Fast Protein Liquid Chromatography (RP-FPLC).

Lectin(300 mg) was dispersed in 2 mL 0.1% (V/V) trifluoroacetic acid (TFA), sonicated, centrifuged and the supernatant (1.9 mg/mL) was filtered and purified on a C18 column (4.6 x 250 mm, 5 µm particles, 300 Å pore size)equilibrated with 0.1% TFA. Adsorbed proteins were eluted using a concentration gradient from 0 to 100% of solution B (80% acetonitrile in 0.1% TFA), represented by the dotted line, at a flow rate of 0.8 mL/min. Absorbance was monitored at 280 nm, at 25 °C, using a FPLC Äkta Purifier UPC-10 system.

Peak 1	NLSLSELDFS	ADTYKSFIKN	LRKQL...
Peak 2	NLSLSELDFS	ADTYK...	

Figure 3.11: Initial N-terminal of the two final peaks eluted in the reversed-phase chromatography.

Amino Acid Abbreviations according to Commission on Biochemical Nomenclature

Abbreviation		Amino acid name
Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartic acid (Aspartate)
Cys	C	Cysteine
Gln	Q	Glutamine
Glu	E	Glutamic acid (Glutamate)
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine
Asx	B	Aspartic acid or Asparagine
Glx	Z	Glutamine or Glutamic acid.
Xaa	X	Any amino acid.
TERM		termination codon

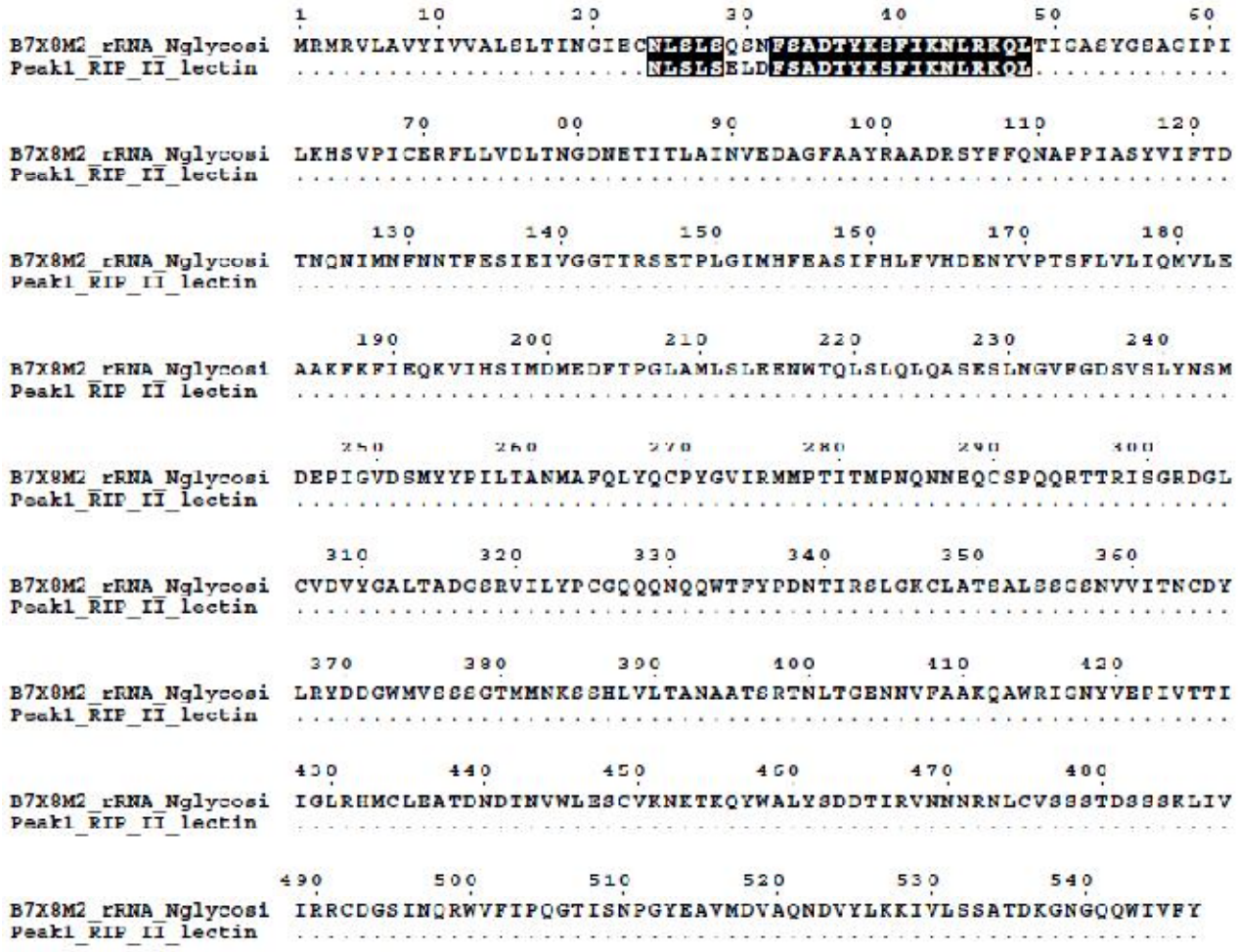

Figure 3.12: Sequence alignment of the initial N-terminal of the peak 1 and MCL1 (Uniprot ID B7X8M2).

The alignment was created by MultAlin(Corpet 1988) and the figure was generated by ESPript (Gouet et al. 1999). The highly conserved residues are in bold.

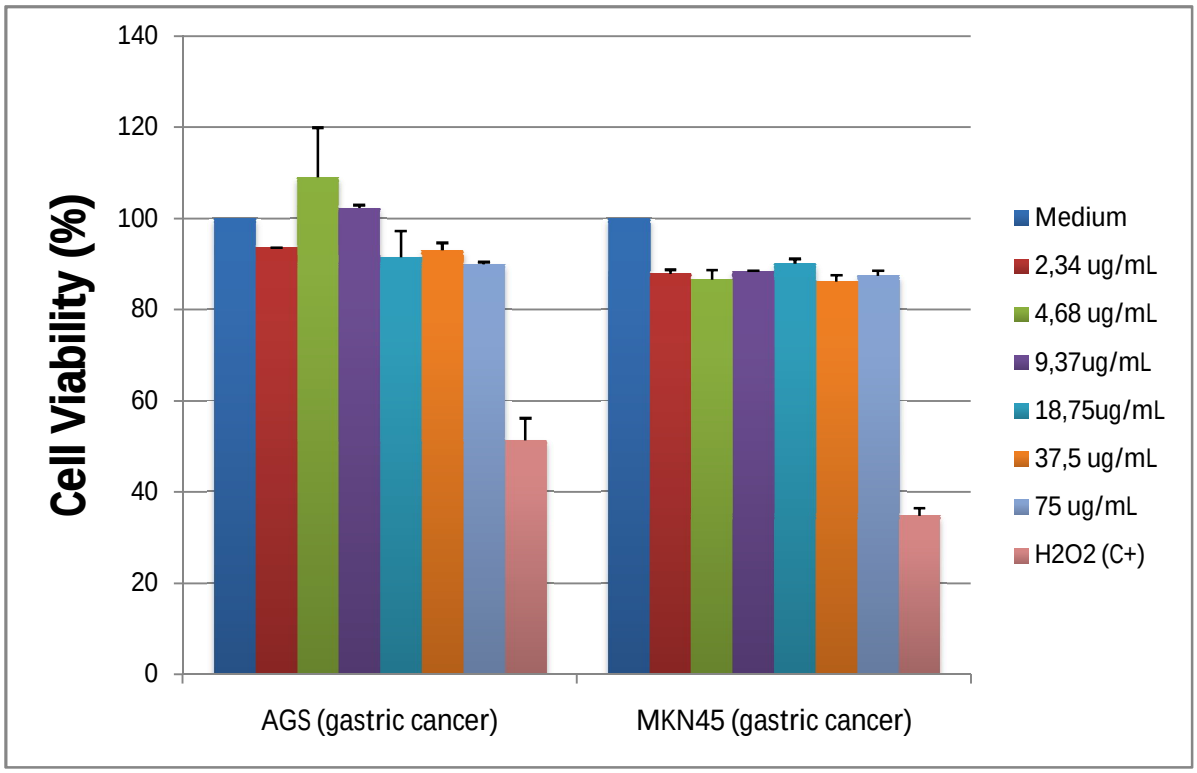

Figure 3.13: Evaluation of Cytotoxicity Using the MTT Assay

Cell viability was assessed on commercial cancer cell lines by using MTT assay, the lectin concentrations ranging from 2,34 µg/mL to 75µg/mL .The absorbance at 550 nm was measured with a plate reader. Absorbance values were blanked against DMSO and the absorbance of cells exposed to medium only (i.e. no lectin) was taken as 100% cell viability (i.e. the negative control of death). For positive control of cell death, cells were alternatively treated with 80 µM of Hydrogen peroxide (H_2O_2).

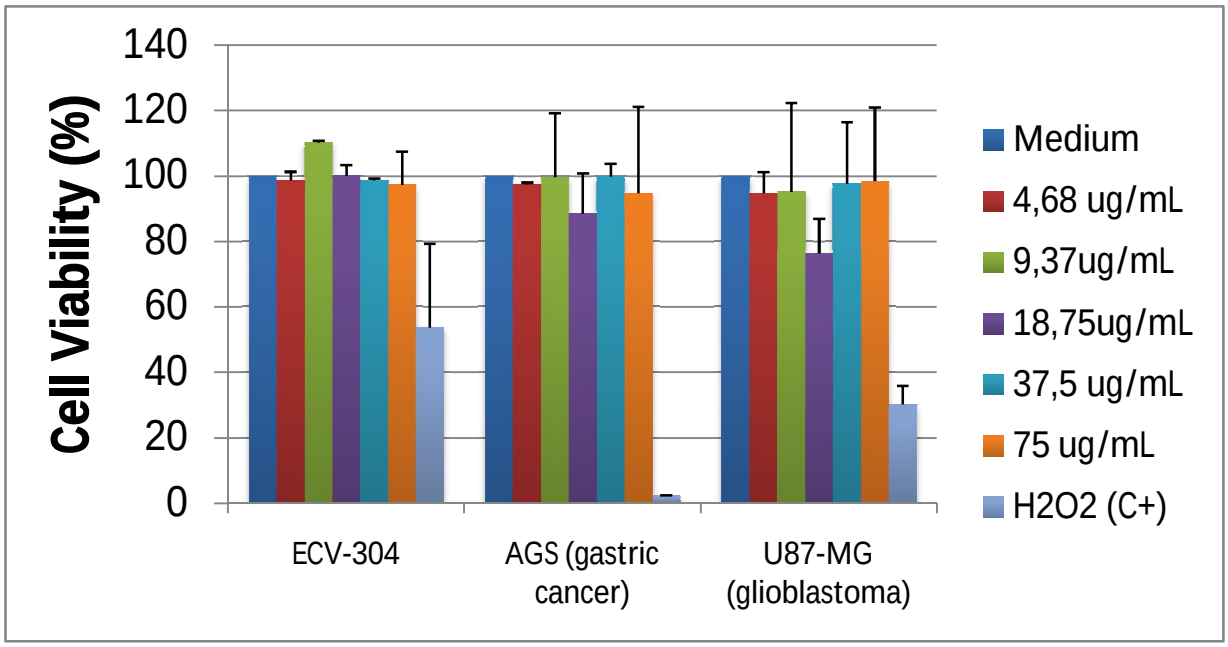

Figure 3.14: Evaluation of Cytotoxicity Using the MTT Assay

Cell viability was assessed on commercial cancer cell lines by using MTT assay, the lectin concentrations ranging from 2,34µg/mL to 75µg/mL. The absorbance at 550 nm was measured with a plate reader. Absorbance values were blanked against DMSO and the absorbance of cells exposed to medium only (i.e. no lectin) was taken as 100% cell viability (i.e. the negative control of death). For positive control of cell death, cells were alternatively treated with 80 µM of Hydrogen peroxide (H_2O_2).