2. Materials and methods

2.1. Materials

2.1.1. Plant materials

Momordica balsamina fruit seeds were brought from urban area of Sudan (Gadrif and north Kurdofan states).

Figure 2.1: Momordica balsamina fruit and seeds.

2.1.2. Erythrocytes

Typed human blood cells (A, AB, B and O) were obtained from healthy donors, while animal blood cells were obtained from the animal house of Sudan University of Science and Technology.

2.1.3. Chemicals

Gel filtration protein markers and alpha agarose lactose affinity matrix were purchased from Sigma Chemicals. All other reagents were either of analytical grade or of highest quality available.

2.1.4. Commercial cancer cell lines

AGS (Human Gastric Adenocarcinoma) andU87-MG (Human Glioblastoma), were obtained from ATCC Company. MKN45 (Human Gastric Cancer) was purchased from Riken BRC Company, while ECV-304 (Human Urinary Bladder Carcinoma) was obtained from Sigma Aldrich Company. All cell lines were available at the department of clinical, toxicological and chemical analysis, Faculty of Pharmaceutical Sciences of Ribeirao Preto, University of Sao Paulo, Brazil.

Cell culture: ECV-304 and MKN-45 cancer cell lines were maintained in RPMI1640 supplemented with 10% Fetal Bovine Serum (FBS) + 2mM Glutamine.AGS and U87MG cancer cell lines were maintained in DMEN supplemented with 10%SFB.

2.2. Methods

2.2.1. Protein estimation

The protein content of the samples obtained during the purification process was determined by method of Lowry et al.(Lowry et al. 1951), using bovine serum albumin as the standard. Readings at 280nm were also used to determine the protein content of the column elutes.

2.2.1.1. Estimation of Protein by UV Absorbance

Principle: Proteins in solution absorb ultraviolet light with absorbance maxima at around 280. Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm.

For estimation sample protein at 280 nm it was necessary to initially prepare a standard curve using BSA (Bovine Serum Albumin) protein standard.

1. (1mg/mL) BSA standard prepared from the given stock (10 mg/mL) in 0.145M NaCl and the graph blotted of quantity of BSA Vs absorbance at 280nm.
2. From the standard BSA 0, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000**µ**g was pipette. Final volume adjusted to 1mL using 0.145M NaCl.

3. The results are tabulated as follow:

	BSA added (µL)	**BSA added (µg)**	**Physiological Saline (µL)**	**OD_{280nm}**
Blank	00	00	1000	To adjust to zero
1	100	100	950	
2	200	200	900	
3	300	300	850	
4	400	400	800	
5	500	500	750	
6	600	600	700	
7	700	700	650	
8	800	800	600	
9	900	900	550	
10	1000	1000	500	
Unknown				

2.2.1.2. Lowry's assay

Principle: The Lowry method is best used with protein concentrations of 0.01-1.0 mg/ml. The method is based on the reaction of Cu^{+}, produced by the oxidation of peptide bonds, with Folin's reagent (a mixture of phosphotungestic acid and phosphomolybdic acid in phenol) in the Folin-Ciocalteu reaction. The reaction mechanism is not well understood, but involves reduction of the Folin reagent and oxidation of aromatic residues (mainly tryptophan, also tyrosine). The concentration of the reduced Folin reagent is measured by absorbance at 660 nm.

Reagents:

1. Lowry A: 2% NaCO3 in 0.1N NaOH
2. Lowry B1: 0.5% CuSO4.5H2O
3. Lowry B2: 1% Sodium Citrate
4. Lowry C (Freshly prepared): 100 mL A+ 1 mL B1+ 1 mL B2
5. Folin-phenol reagent (diluted 3 times)
6. Standard curve was prepared as exactly described for protein estimation at 280nm then protein quantity calculated in the given unknown sample. Standard BSA: 1mg/ mL.Working: 100µg/ mL.

	Working/Sample (mL)	**2D H20 (mL)**	**Lowry C (mL)**	**Incubation (min)**	**Diluted Folin (mL)**	**Incubation (min)**	**O. D At 660 nm**
Blank	0.0	1	3	15	0.5	45	
1	0.2	0.8	3	15	0.5	45	
2	0.4	0.6	3	15	0.5	45	
3	0.6	0.4	3	15	0.5	45	
4	0.8	0.2	3	15	0.5	45	
5	1.0	0.0	3	15	0.5	45	
Unknown							

2.2.2. Preparation of seed extract

The extraction was carried out as described by Konozy et al.,(Konozy et al. 2002). Season fresh, mature, and good quality seeds of *M. balsamina* seeds were ground to a fine flour in a coffee grinder and the meal (100 g) was defatted with petroleum ether in a ratio of (5mL/1g powder). The ether layer was removed by filtration

system. Soluble protein was precipitated with equal amount of chilled acetone (added drop wise with continuous stirring). The extract was filtered through filtration system which used for sterilization, and further dehydrated extensively with chilled acetone to get acetone dried powder. Acetone dried powder was extracted for 4 hours with 0.145 mM NaCl at 4^{o} C (in a ratio 5:1). The whole extract was filtered through filter paper and then centrifuged for 45 min at 6000*rpm* at 4^{o} C.

2.2.3. Purification of lectin on alpha agarose lactose matrix

This was carried out essentially as described by Konozy et al.,(Konozy et al. 2003)In a syringe of 10mL capacity, 2mL of alpha agarose lactose were loaded; column was initially washed with 100 mL of 100 mM acetate buffer pH 5, equilibrated with 0.145M NaCl. Protein was loaded into the column; recycled for several times to ensure maximum retention of lectin on matrix. Unbound proteins were washed off with equilibration 1M saline till reading at OD_{280nm} dropped to $\leq$0.2. Elution of bound lectins was done by 200 mM lactose; 3 ml fractions were collected at reduced flow rate of 3mL/min. Fractions were read for protein content by spectrophotometer at 280 nm. Fractions that exhibited ODs above 0.06 were pooled, precipitated by ammonium sulphate 100%, then dialyzed against distilled water and tested for lectin activity.

2.2.4. Hemagglutination inhibition

Hemagglutination test was conducted in a microtiter plates, in a final volume of 100µl. Each well contained 50µl of lectin solution and 50µl of 4% (v/v) suspension of either untrypsinized or trypsinized erythrocytes. Agglutination was assessed after incubation for 30 minutes at room temperature. Hemagglutinating activity was expressed titer, namely, the reciprocal of the highest dilution that gave a

positive result.(Konozy et al. 2003) The specific hemagglutinating activity was defined as titer (per mg lectin). Type O blood group was used throughout out this study.

2.2.5. Carbohydrate content estimation

The neutral sugar content of the lectin was estimated by the phenol- sulfuric acid method using glucose as standard (Dubois et al. 1951).

Reagents: Standards: glucose 1 mg/ml stock solution,0.1mg/ml working solution, 5% phenol, 96% sulphuric acid.

Procedure:

w/ standard (ml)	D.W (ml)	Phenol(ml)	Sulphuric acid(ml)
0	1.0	1.0	5.0
0.2	0.8	1.0	5.0
0.4	0.6	1.0	5.0
0.6	0.4	1.0	5.0
0.8	0.2	1.0	5.0
1.0	0	1.0	5.0

Tubes were incubated for 30 minutes in room temperature then absorbance read at 490nm.

2.2.6. Molecular mass determination

The native molecular mass of the *Momordica Balsamina* lectin was determined by gel filtration, whereas estimation of the subunit molecular weight was done using sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE).

2.2.6.1. Gel-filtration chromatography

The molecular weight of the intact lectin was loaded on Sephadex G-100 column (1.5Cm× 64Cm) previously equilibrated with 0.145 M NaCl, and the protein was eluted at 1.5 ml/min with the same buffer. Protein elution was monitored at 280nm. Molecular weight was determined by calibrating the column against standard molecular weight markers.(Irvine 1994)

Briefly: the eluted fractions were tested for hemagglutinating activity then the fractions with positive activity were further investigated for both specific and unit activity, the highest results represents the elution volume (Ve) which then divided by the void volume (Vo) of blue dextran. Then the molecular weight obtained after using the formula from the standard molecular weight markers curve.

2.2.6.2. Sodium dodecyl sulfate polyacrylamide electrophoresis

Molecular mass of the monomeric form was estimated by reducing- sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli 1970), by comparing with electophoretic migration of standard molecular mass using Spectra Multicolor Broad Range Protein Ladder (10 to 260kDa), from Thermo Scientific. Protein bands were stained with Commassie Brilliant R-250.

SDS-PAGE preparations performed in room temperature as follow:

12.5% resolving gel: 4.2 ml of 30% acrylamide**,** 3.1 ml of d.H_2O**,** and 2.5 ml of resolving gel buffer (1.5M Tris-base pH 8.8)

, 100 µl 10% SDS, 10 µl of TEMED, 100 µl of ammonium persulphate (APS). Polymerization was done for about 1 hour.

4% stacking gel: 1.3 ml of 30% acrylamide**,** 4.0 ml d.H_2O**,** 2.5 ml of stacking buffer (1M of Tris-HCl pH 6.8)**,** 100µl 10% SDS**,** 10 µl of TEMED **,** 100 µl of APS.

Sample buffer: 4ml d.H_2O,0.05 M Tris-OH 1ml**,** 0.8 ml glycerol**,** 1.6 ml of 10% SDS, 0.4ml of β-mercaptoethanol, 0.2 ml of 0.05% bromophenol blue.

Running buffer pH 8.3: Tris-OH 3.02g, 14.4g glycine, 10% SDS, 100ml of d. H_2O

Staining: 23ml glacial acetic acid**,** 114 ml of methanol**,** 0.63g of Coomassie Brilliant Blue powder stain**.**Volume completed to 250ml.

De-staining:10 ml glacial acetic acid**,** 80 ml of methanol**,** and 100 ml of d.H_2O.

2.2.7. Lectin sugar specificity

The sugar specificity of the lectin was tested as described by Konozy et al., (Konozy et al. 2002) by inhibiting the hemagglutinating activity using 200mM of 7 different sugars (Arabinose, Glucose, Galactose, Mannose, Maltose, Sucrose and Lactose). Results were expressed as the minimal concentration of sugar which effectively inhibited hemagglutinating units of lectin.

2.2.8. Effect of temperature on hemagglutinating activity

Heat stability was determined by incubating lectin 0.03 mg/ml at 20, 30, 40, 50, 60, 70, 80 and 90 ºC for 30 minutes. The samples were immediately cooled and hemagglutinating activity was determined as described by Konozy et al., (Konozy et al. 2003)

2.2.9. Effect of pH on hemagglutinating activity

This was performed as described by Konozy et al.,(Konozy et al. 2003) by incubating fixed concentration of the lectin 0.03 mg/ml with buffers of varying pH (between pH 2 to pH 13 prepared by HCL and NaOH, then the pH monitored by pH meter) at a total volume of 100µl for 1 h at room temperature (25–27 °C). The pH of lectin samples was adjusted to pH 7.0 by addition of 0.1 N NaOH or 0.1 N HCl before hemagglutinating activity was examined.

2.2.10. Effect of denaturing agent on hemagglutinating activity

The effect of the denaturing agent was tested by incubating of fixed concentration of lectin 0.03mg/ml with a wide array of urea concentrations ranging from 1 to 8M for 2 hours before testing for remaining hemagglutinating activity.

2.2.11. N-terminal sequencing

2.2.11.1. Reversed-phase Fast Protein Liquid Chromatography (RP-FPLC) using a C18 column

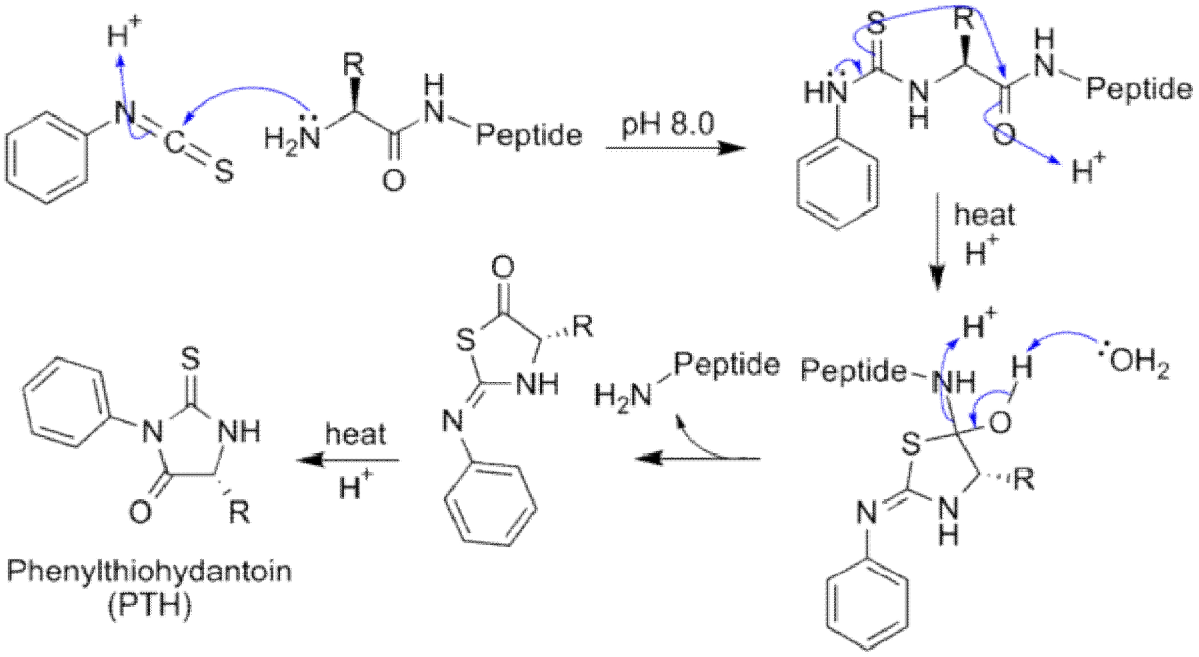
Fast protein liquid chromatography (FPLC) is a form of liquid chromatography that is often used to analyze or purify mixtures of proteins. As in other forms of chromatography, separation is possible because the different components of a mixture have different affinities for two materials, a moving fluid (the "mobile phase") and a porous solid (the stationary phase). In FPLC the mobile phase is an aqueous solution, or "buffer". The buffer flow rate is controlled by a positive-displacement pump and is normally kept constant, while the composition of the buffer can be varied by drawing fluids in different proportions from two or more external reservoirs. The stationary phase is a resin composed of beads, usually of cross-linked agarose, packed into a cylindrical glass or plastic column.

Reversed-phase chromatography (also called RPC, reverse-phase chromatography, or hydrophobic chromatography) includes any chromatographic method that uses a hydrophobicstationary phase. Any inert non-polar substance that achieves sufficient packing can be used for reversed-phase chromatography. The most popular column is an octadecyl carbon chain (C18)-bonded silica.

Lectin (300 mg) was dispersed in 2 mL 0.1% (V/V) trifluoroacetic acid (TFA), sonicated in a bath-sonicator (Thornton, INPEC Eletrônica, Vinhedo, SP, Brazil) for 10 minutes, centrifuged at 15,700 *x g*, 4 ºC, for 10 minutes and the protein concentration was estimated by Nano Drop 2000 (Thermo Scientific, Wilmington, DE, USA). The supernatant (1.9 mg/mL) was filtered through a 0.22-µm membrane (Merck Millipore, Darmstadt, Germany) and applied on a C18 column (4.6 x 250 mm, 5 µm particles, 300 Å pore size, Shimadzu Co., Kyoto, Japan) equilibrated with 0.1% (V/V) trifluoroacetic acid (TFA). Adsorbed proteins were eluted with a step 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. The absorbance was monitored at 280 nm, at 25 °C, using a FPLC Äkta Purifier UPC-10 system (GE Healthcare, Uppsala, Sweden).

Edman degradation mechanism:

Edman degradation, developed by Pehr Edman, is a method of sequencingamino acids in a peptide. In this method, the amino-terminal residue is labeled and cleaved from the peptide without disrupting the peptide bonds between other amino acid residues.



Phenylisothiocyanate is reacted with an uncharged terminal amino group, under mildly alkaline conditions, to form a cyclical phenylthiocarbamoyl derivative. Then, under acidic conditions, this derivative of the terminal amino acid is cleaved as a thiazolinone derivative. The thiazolinone amino acid is then selectively extracted into an organic solvent and treated with acid to form the more stable phenylthiohydantoin (PTH)- amino acid derivative that can be identified by using chromatography or electrophoresis. This procedure can then be repeated again to identify the next amino acid. A major drawback to this technique is that the peptides being sequenced in this manner cannot have more than 50 to 60 residues (and in practice, under 30). The peptide length is limited due to the cyclical derivatization not always going to completion. The derivatization problem can be resolved by cleaving large peptides into smaller peptides before proceeding with the reaction. It is able to accurately sequence up to 30 amino acids with modern machines capable of over 99% efficiency per amino acid. An advantage of the Edman degradation is that it only uses 10 - 100 pico-moles of peptide for the sequencing process. The Edman degradation reaction was automated in 1967 by

Edman and Beggs to speed up the process and 100 automated devices were in use worldwide by 1973.

The eluted peaks from RP-FPLC were lyophilized, dispersed in water and submitted to Edman degradation (Edman & Begg 1967), on an automated sequencer model PPSQ-33A (Shimadzu Co., Kyoto, Japan). The search for sequential identities was carried out by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

BLAST(Basic Local Alignment Search Tool) is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences. A BLAST search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold.

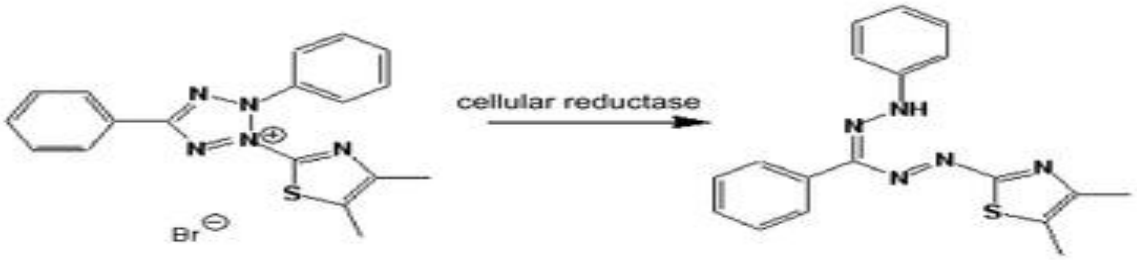
The BLAST program can either be downloaded and run as a command-line utility "blastall" or accessed for free over the web. The BLAST web server, hosted by the NCBI, allows anyone with a web browser to perform similarity searches against constantly updated databases of proteins and DNA that include most of the newly sequenced organisms. There are now a handful of different BLAST programs available, which can be used depending on what one is attempting to do and what they are working with. These different programs vary in query sequence input, the database being searched, and what is being compared.

BLAST is actually a family of programs (all included in the blastall executable). One of them is: Protein-protein BLAST (blastp): This program, given a protein

query, returns the most similar protein sequences from the protein database that the user specifies.

2.2.12. Evaluation of cytotoxicity of lectin using the MTT assay

MTT assay: The MTT assay is a colorimetric assay for assessing cell viability. NADPH-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color Tetrazolium dye assays can also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferative to resting status) of potential medicinal agents and toxic materials. MTT assays are usually done in the dark since the MTT reagent is sensitive to light. MTT, a yellow tetrazole, is reduced to purpleformazan in living cells. A solubilization solution (usually either dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergentsodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer.



In vitro cytotoxicity of lectin was assessed by monitoring the conversion of MTT

(1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan, Sigma-Aldrich, MO, USA) to formazan. The reduction of MTT is catalyzed by mitochondrial dehydrogenase enzymes and is therefore a measure for cell viability (Mosmann, 1983).

Briefly, AGS (Human Gastric Adenocarcinoma), MKN45 (Human Gastric Cancer), U87-MG (Human Glioblastoma)and ECV-304 (Human Urinary Bladder Carcinoma) cell lines were seeded into 96 well microtiter plates and allowed to adhere for an overnight period ($1x10^5$ cells/ well - 200 µl). The next day, the medium was removed from the wells and replaced with filter sterilized complete medium containing the lectin concentrations ranging from 2,34µg/mL to 75µg/mL (the test was done in triplicate).The cells were then incubated with the lectin for 20h. Then, media containing the lectin was removed and replaced with complete media. Cell viability was assessed by adding 20 µl of filter sterilized MTT (5 mg/ml in PBS) to each well. Following a 4 h incubation period with MTT, media was removed with a needle and syringe and the blue formazan crystals trapped in cells dissolved in 100 µl of sterile DMSO (Dimethyl Sulfoxide, Sigma-Aldrich) by incubating at 37 ºC for 30 min. 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).