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Purification and Characterization of Novel Lectin from the Georgian Endemic Plant Polygonatum Obtusifolium Miscz. with Anticancer Activity

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Abstract:- A novel Polygonatum Lectin (GPL) was isolated from the rhizomes Georgian endemic plant Polygonatum obtusifolium Miscz. using a combination of gel-filtration chromatography on Toyopearl HW-55 column, affinity chromatography on glutaraldehyde fixed rabbit erythrocyte column and conventional protein purification techniques. GPL had a special agglutinating activity with rabbit trypsin-treated erythrocytes at a minimum concentration of 0.000095 mg/ml. Using the marker proteins and analytical gel-filtration, molecular weight of the native GPL was established, which corresponded to 30 kDa. Using electrophoresis of marker proteins on polyacrylamide gradient gel (10-25%), in the presence of SDS, it has been showed that GPL dissociated into subunits, migrates as a single band and its molecular mass corresponds to 15 kDa. Thus it was concluded that GPL consists of two same subunits and has homodimer quaternary structure. MTT analyses method showed growth inhibition effect of GPL on short- term (48 h) primary cancer cell culture derived from human skin, lung, ovarian and breast cancer. Carbohydrate α-methyl-mannopyranoside inhibited cytotoxic and cancer cell agglutination activity of GPL. Cancer cell agglutination and growth inhibitory effects of various concentration of GPL were lost at the corresponding level with the lose of mannose-binding activity, indicating that there may be a close link between the agglutinating activity and anti- proliferative property of GPL. It was suggested that cytotoxic effect of GPL on cancer cells should be mediated by its specific interaction with α-methyl-mannopyranoside containing cell membrane surface receptors inducing destruction of cancer cells.

Keywords:- *Polygonatum obtusifolium Miscz. rhizomes, mannose-specific lectin, isolation, properties, anticancer activity.*

I. INTRODUCTION

Physiologically active compounds of plant origin are widely used in biology, agriculture, medicine and other fields of science. Among them are the sugar binding proteins lectins, which are given special attention last time [1, 4, 6].

Proteins with the ability to selectively and reversibly bind carbohydrates of glycoconjugates, causing agglutination of cells, are considered to be lectins. Nowadays lectins are successfully used as research tools and affinity sorbents for identification of glyco- conjugates, cells and organelles. As specific biological probes they are successfully applied for the research of terminal membrane carbohydrates. So, isolation of new plant lectins and ascertaining their biological role still remains one of the topical bio -medical problems. Therefore, we made our purpose to identify and study lectins from Georgian endemic medicinal plant Polygonatum obtusifolium Miscz. Polygonatum obtusifolium (C. Koch) Miscz. ex Grossh (King Solomon's-seal, Solomon's seal), which belongs to the family of Convallariaceae [3].

However little is known about the biologically active proteins activity towards cancer cells [17, 18]. The present work aims to identify lectins in Georgian endemic medicinal plant Polygonatum obtusifolium Miscz and study some biochemical properties and its antitumor activity.

II. MATERIALS AND METHODS

- *Investigation Object*

Different parts of plants (stems, leaves, flowers, seeds) and underground parts (roots, rhizomes, buds) of the Georgian endemic plant, mountainous Solomon's Seal (Polygonatum obtusifolium) were used as object of investigation. Plant material was harvested in autumn and in spring, at the stage of active growth and development of the plant.

- *Exstraction of Lectins from Different Parts of the Plant*

Serapate underground and aerial parts of Polygonatum plant were homogenized. Extraction of soluble protein was made by solution: 0.9% NaCl, 0.1% □-mercaptoethanolamin

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(□-M), 0.04M K+ phosphate buffer, pH 7.4; ratio between the raw material and extracting solution was w/v=1/5. For the maximum extraction of soluble proteins the homogenate was placed to the magnetic stirrer for 30 minutes in room temperature (20-25ºC). The extract was filtered through the double gauze and the filtrate was centrifuged at 16000 r/m for 15 minutes. The supernatant was filtered through the Whatman CF/C and Sinpor-0.45-0.22□M filter. Excess inorganic ions were removed by dialysis on G-10 Sephadex column (50x2.7 cm). Extracts were kept at +4ºC.

Hemagglutination activity was determined visually using 96-well immunological microtiter U- plates using rabbit trypsin-treated erythrocytes with the method of Takatsy [16]. This was performed with Takatsy microtitrator using 50 µl loops and 50 µl 2% erythrocytes suspension, 50 µl normal saline and the serially diluted agglutinin were mixed and scored for incubated for 1 h. Lectin hemagglutinating activity (HA) was estimated according to the minimum protein concentration (mg/ml), which causes full agglutination of rabbit trypsin-treated erythrocytes. Lectin specific activity (SA), which is its inverse value and reflects the minimum concentration of proteins which caused agglutination: SA=T-1xC-1, where T- (titer) is the degree of minimum protein concentration in which agglutination is still noticeable, C - is protein concentration, expressed in mg/ml.

Lectin content (LC) was judged by the ratio of overall protein content to lectin activity (conventionally agglutination unit, hemagglutination unit – HU) or by the formula GAA=1/Tm (T - titer of lectin hemagglutinating activity; m - mass of the used fragment of Polygonatum obtusifolium. Kinetics of hemagglutination activity was measured by the photocolorimetric method, at 670 nm light, a 200 µl 2% suspension of trypsin-treated erythrocytes was introduced into the control and test cuvettes. Lectin fraction was added to the testing cuvettes and PBS of the same volume to the control one. In the control cuvette light transmission at 670 nm was considered as 100% and variation of this index was fixed with an recorder, connected with a photo colorimeter (КФК-3) [1].

➢ *Purification of Lectin (GPL)*

For the extraction of lectins, rhizomes of Solomon's seal were homogenized in a homogenizer of a blender type. Soluble protein fraction was extracted using the extraction solution of the following composition: 0.9% NaCl, 40mM K+-phosphate buffer, pH 7.4, 0.1% □-mercaptoethanol; extraction was made in conditions of (w/v = 1/5) ratio of raw material at the pH 7.4. The homogenate was placed on magnetic stirrer for 30 minutes at room temperature. Extract was filtered through the double gauze and the filtrate was centrifuged at 15000 r/min for 15 minutes. For the partial purification and fractionation of lectin the proteins were precipitated with ammonium sulphate in conditions of 0-60% saturation. Suspension of proteins was centrifuged at 8000 r/min for 5 minutes. The supernatant was removed by decantation and the precipitated proteins dissolved in the minimum volume of PBS. Protein solution was dispersed in the homogenizer and centrifuged at 8000 r/min for 10 min. The supernatant was collected by decantation and dialyzed chromatographically on G-10 column (1.6x40 cm) to remove the ammonnium sulphate. The dialyzed protein fraction was placed at 60ºC temperature for 15 min, to remove the thermolabile proteins. The protein solution was placed in icy bath for 30 min and centrifuged at 18000 r/m for 10 min.

➢ *Treatment with Acetone*

The sediment of proteins was removed and the precipitated with 5 volumes of cold acetone. The mixture was centrifuged at 3000 r/min per 15 minutes and the sediment dissolved in the minimum volume of PBS. The proteins labile to aceton were removed by centrifugation at 3000 r/min for 15 min and was further purified.

Lectin specificity to carbohydrates was studied by means of hapten-inhibitory method [9]. For the analysis we used 0.6 M solution of monosaccharide's prepared on PBS. In the experiments 18 different carbohydrates were used: D-galactose, methyl-D-galactose, α-methylmanno- pyranoside, D-mannose, D-raffinose, D-glucose, D-ramnose, N-acetyl-D-glucosamine, N-acetyl- D-galactoseamine, D-galacturonic acid, D-fructose, L-inosite, D-arabinose, L-ribose, Melibiose, D-lactose, D-cellobiose, Saccharose. Sugar solution was tittered from 200 mM with decreasing concentration, on the immunological plates. Equal concentration of 1:4 titer lectin solutions was introduced in all cells of the plate. Hapten specificity was estimated by minimal concentration of sugar (mM) which resulted inhibition of lectins hemagglutination.

➢ *Gel-Filtration on Toyopearl HW-55 Column*

Further purification of the protein fraction with lectin activity was carried out on Toyopearl HW-55 column (3.57x70cm), which was balanced with PBS. Chromatograhy was preformed using the HPLC (Knauer). For elution was used PBS. Elution rate was 2 ml/min. Detection was done at 280 nm vawe length. Protein fractions, eluted from the column were tested on lectin activity. Purification process of GPL by the affinity chromatography on glutaraldehyde fixed rabbit erythrocytes physically entrapted in Biogel P-150. Further purification of proteins, possessing lectin activity was performed using the method of affinity chromatography on the column with trypsin-treated rabbit erythrocytes, fixed by glutaraldehyde (1.60x20 cm) using the HPLC (LKB). Elution rate was 0.5 ml/min. Detecton was carried out at 220 nm vawelength. Elution of the lectin, immobilezed on the sorbent of affine column, balanced with saline was perfomed using the acidic solution (40 mM Glycine-HCl, pH 3.5) (solution A), but in control experiments this was done using the saline solution of lectin- specific carbohydrate 50 mM D-mannose (PBS, pH 7.4) (Solution B). Protein fraction, eluted with the Solution-A was dialysed against PBS, while the protein fraction, eluted with the Solution-B was dialysed with acidic solution, for 24 hours at +4ºC temperature Lectins were precipitated with ammonium sulphate by the salting out

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method (0-60%). The precipitate of salted out proteins was dissolved in minimum volume of PBS and centrifuged at 8000 r/min for 10 minutes. Dialysate, possessing lectin activity was dialysed and used in further researches.

- *Preparation of the Glutaraldehyde Fixed Rabbit Erythrocyte Chromatographic Column*

With the aim of fixing rabbit erythrocytes with glutaraldehyde the erythrocytes were treated with trypsin using the above described method. Trypsin-treated erythrocyes 2% suspension was prepared in 2.5 or 5% solution of gutaraldehyde, agglutination buffer (pH 7.4) and placed on the stirrer for 17 hours at 4ºC. The percipitate was washed in quadriple volume of agglutination buffer, centrifuged (700 g, 10 min), determined hematocrite and 2% suspension of erythrocytes was prepared in 1M glycine solution (pH 7.4), prepared on agglutination buffer. The suspension was stay on a stirrer for 17 hours at +4ºC. The precipitate was washed off in a quadriple volume of agglutination buffer and centrifuged (700 g x10 min); after the determination of hematocrite the sediment was mixed with Biogel P-150 at 1:10 ratio and the affine column (75x20 mm). The column, fixed with glutaraldehyde was prepared. The column was well washed, first, by agglutination buffer and, then, with 0.2M glycine/HCl buffer (pH 3.0). Before the use the column was balanced with agglutination buffer (PBS).

Determination of GPL molecular weight by the method of gel-filtration. For the determination of the protein, purified by affine chromatography, the HPLC system on the Toyopearl HW-55 column (1.0x60 cm), equilibrated with PBS was used. Chromatography was carried out in the following conditions: elution rate - 1.0ml/min, detecton was performed at 280 nm wave length. For the standard and experimental protein fractions distribution coefficients were determined by the following formula: Kav=(Ve-Vo)/(Vn-Vo). The following standard proteins were used: Albumin (66,000 Da), Carbonic Anhydrase (29,000 Da) and Cytochrom C (12,400 Da) (Sigma Gel Filtration Molecular Weight Markers Kit for Molecular Weights 12,400–200,000 Da).

Analytical electrophoresis of GPL in dissociated conditions. Electrophoresis of proteins was carried out in dissociated state, using the system of Laemmli on 2mm thick 10-25% polyacrylamide gradient gel in the presence of 0.1% SDS [8]. Elecrophoresis lasted 3.5 hr (on 1 ml gel at 2mA amperage. The following low molecular weight marker proteins were used for the establishing of molecular weight (SIGMA - Molecular Weight Marker KIT, For Molecular Weight Range 14,000–70,000 Da): Albumin, Bovine- 66, 000; Ovalbumin, Chicken-45,000; Pepsin, Porcine Stomach Mucosa-34,700; Trypsinogen, Bovine, PMSF treated-24,000; β- Lactoglobulin, Bovine-20,100; Lysozyme, Egg White-14,200.

- *Antitumor Activity of GPL*

The cytotoxic effect of the GPL on the human malignant tumors has been studied in vitro on the short-term primary cultures derived directly from the parent tissue of human skin, lung, ovarian and breast cancer. The tumor tissue was taken from the untreated patients subjected to the surgery. For the separation of cells the tissue was treated mechanically and then disaggregated with enzymes. The suspension of the separated cells was prepared on RPMI 1640 area, to which fetal bovine serum (10%) and gentamicin (50μg/ml) were added. Cells were seeded into 96-well microplates (cancer cells = 5×104 cells/well). For the determination of the cell viability we used 0.2% solution of the trypan blue, a vital dye stipulated in the project. In the experiments without GPL-1 the cell viability was in the range of 90-95%. All manipulations were carried out in sterile environments. The cells were cultivated on the microtiter plates with 96 wells in conditions of three different concentration of GPL-1 (10, 50,100 μg/ml). In the control wells nutrient medium of standard quantity was placed. The anti- cancerogenic and control experiments were conducted in triplicate for each concentration of GPL. The incubation was carried out at 37°C during 48 hours in the conditions of humidity and 5% of CO2. The cytotoxic effect was estimated by means of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test [15]. after 3-hour-incubation. For the extraction the solution consisting of SDS (10%), isobutanol (50%) and HCI (0.01 N) was used. The extinction index was determined with Biotek EL 312 counter at the wavelength of 570 nm.

Inhibition of the cytotoxic activity of GPL with the α-methyl-mannopyranoside. Initial solution of the GPL (1 mg/ml) was added by equal quantities – 400 mM α-methyl-man- nopyranoside and was incubated on the shaker in the thermostat at 37°C temperature for 3 hours. After incubation, the free molecules of α-methyl-mannopyranoside, unbound with GPL, were removed by means of dialysis carried out in dialysis packs during the night. For sterilization the dialyzate was filtered through the filters with 0.22 mμ pore diameter (Millipore) and were tested on hemagglutination activity towards the trypsin treated rabbit erythrocytes, and, then on cytotoxicity towards the cancer cells in short-term suspension cultures in vitro. Protein concentration was measured by the method of [10]. All experiments were performed in triplicate. Statistical analysis was performed using Student's t-test and p-values < 0.05.

III. RESULTS AND DISCUSSION

- *Lectin Distribution in Aerial and Underground Plant Parts of Polygonatum*

In the first series of experiments were studied plant aerial (stem, leaf, flower and seeds) and underground parts of Polygonatum (root, rhizome and bud) on the content of lectins. Result showed that only underground part crude extracts harvested in spring had haemagglutination activity.

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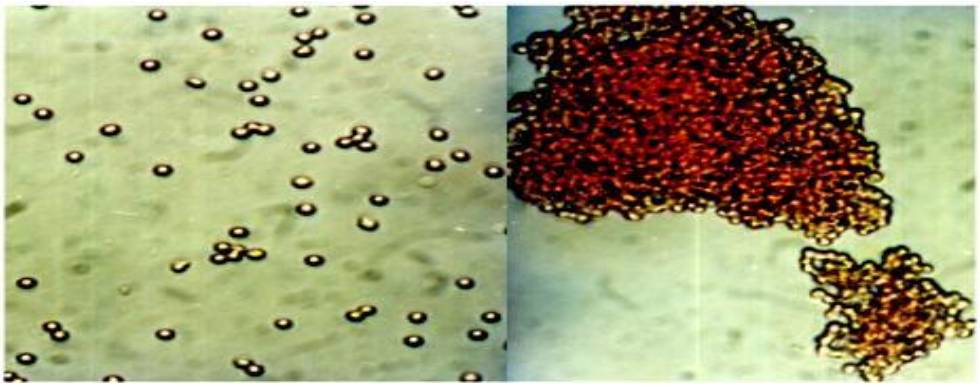

Fig. 1:- GPL stimulated haemaglutination of trypsin treated rabbit erythrocytes under light mic- roscope. 1. control – erythrocytes, 2. test - erythrocytes+GPL

In the next series of experiments was studied localization and distribution of GPL in different underground parts of plants (root, rhizome and bud) in differing physiological condition (Table 1). As seen from the Table, protein content is the highest in the extract of autumn bud. At the same time the highest values of such lectin indexes as T, HA, SHA and LC were registered in extracts of root and rhizome. In particular these values are 5 times higher in root extracts and 10 times higher in rhizome extracts as compared with bud extract.

Harvested in Autumn					
Underground parts of Polygonatum	C mg/ml	T	HA mg/ml	SHA mg/ml	LC
Root	0.85	2^5	0.007	37.6	607
Rhizome	0.76	2^6	0.003	84.2	1260
Bud	0.98	2^3	0.031	8.16	158
Harvested in Spring					
Root	1.56	2^4	0.070	10.25	111.4
Rhizome	1.63	2^5	0.045	19.63	181.1
Bud	1.70	2^2	0.080	2.35	106.2

Table 1:- Protein content (C), haemagglutination titre (T), haemagglutination activity (HA), specific hemagglutionation activity – (SHA) and lectin content (LC) in different extracts of underground parts of plant (root, rhizome and bud) depending on physiological condition of plants harvested in spring and in autumn.

As is seen from the table in spring, in the period of active vegetation and growth, the content of C, T, HA, SHA and LC was almost 2 times higher in underground parts of Polygonatum plant, as compared with autumn period.

➢ *Carbohydrate-Binding Specificity*

In a special series of experiments it was studied specificity of GPL in relation to different carbohydrates (Table 2). To get full-value characteristics of lectins it is necessary to ascertain lectins specificity to carbohydrates. Lectins are known to bind with carbohydrates specifically and inhibit of lectin-induced haemagglutination. As shown in table 5 were tested 18 different carbohydrates at an initial concentration of 200 mM.

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Carbohydrates (Initial concentration 200 mM)	Inhibition of Haemagglutination Activity	Minimal inhibiting concentration (mM) of a carbohydrate
D-galactose	-	
α-methyl-D-galactose	-	
α-methylmannopyranoside	+	25
D-mannose	+	50
D-raffinose	-	
D-glucose	-	
D-ramnose	-	
N-acetyl-D-glucosamine	-	
N-acetyl-D-galactoseamine	-	
D-galacturonic acid	-	
D-fructose	-	
L-inosite	-	
D-arabinose	-	
L-ribose	-	
Melibiose	-	
D-lactose	-	
D-cellobiose	-	
Saccharose	-	
Chitin	-	

Table 2:- The influence of carbohydrates on haemagglutination activity of GPL + inhibition of haemagglutination activity. _ lack of inhibition haemagglutination activity.

The data presented in the table demonstrate that lectin GPL haemagglutination activity was inhibited only in the presence of mannose and α-methylmannopyranoside. It indicate that the lectin GPL isolated from Polygonatum obtusifolium rhizome is mannosespecific lectin

➢ *GPL Purification and Some Characteristics*

The main stages of purification of lectin GPL isolated form the rhizomes of Solomon's seal, are presented in the Table 3. The process of purification of lectin, isolated from Solomon's seal rhizome was consisted of 7 stages: 1. Isolation of protein extract from the rhizome; 2. Chromatography on the Toyopearl HW-55 column; 3. Fractionation of GPL containing proteins with ammonium sulphate; 4. Thermal treatment at +60oC for 30 min; 5. Treatment with acetone; 6. Chromatography on the Toyopearl HW-55 column; 7. Affinity chromatography on the column, stabilized with glutaraldehyde.

Main stages of purification of GPL	Haemagglutination activity (mg/ml)	Purification degree
Extract	0.075	0
Chromatography	0.0080	9
Fractioning with ammonium sulphate	0.0045	17
Thermal treatment	0.0030	25
Treatment with acetone	0.0010	75
Chromatography	0.0008	94
Affine chromatography	0.000095	789

Table 3:- Main stages of purification of GPL.

Table 3 showed that after each stage of purification haemagglutination activity increased correspondingly 17, 25, 75, 95 times and after the final step activity of GPL corresponding0.000095 mg/ml, and purification degree attained 789.

As seen from the Fig 2, 9 different protein fractions were extracted from the rhizome on the Toyopearl HW-55 column. Haemagglutination activity was detected only in 3rd protein peak. Haemagglutination activity of the mentioned protein corresponds 0.0080 mg/ml.

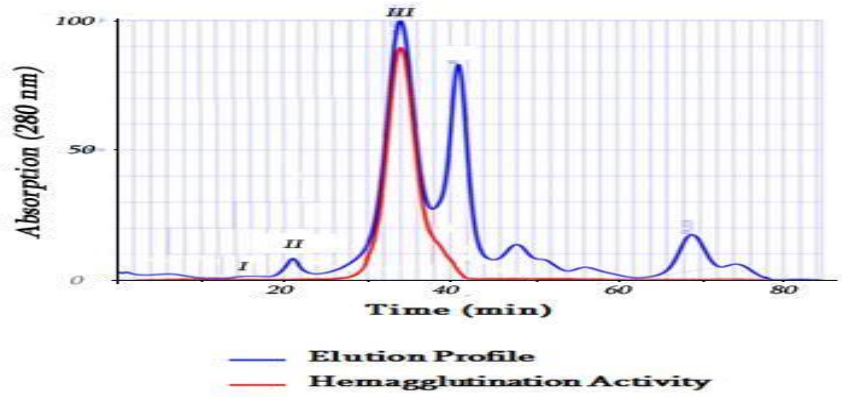

Fig 2:- Elution profile on the Toyopearl HW-55 column Polygonatum rhizomes crude extract

At the final stage of purification of GPL the method of affine chromatography was used. Column of glutaraldehyde fixed trypsin-treated rabbit erythrocytes was used as absorbent (Fig.3).

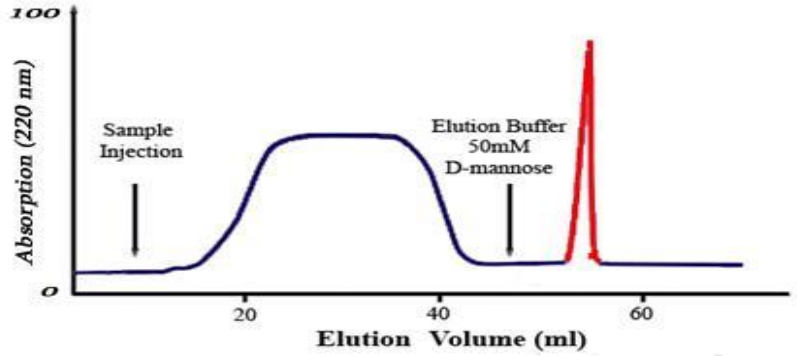

Fig. 3:- Elution profile of affinity chromatography of GPL on the column glutaraldehyde fixed trypsin-treated rabbit erythrocytes. Unbound ballast proteins; Hemagglutination activity

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As seen from the Fig 3, after running the lectin-containing protein fraction through the column of trypsin-treated erythrocytes, the fraction of ballast proteins, unbound with the column was eluted with PBS, which did not had haemagglutination activity. Elution of the protein fraction with haemagglutination activity from the column was performed using the GPL specific carbohydrate, 50 mM D-mannose-containing PBS solution and its haemaggutination activity corresponds 0.000095 mg/ml. According to calculations, done with the use of the calibration curve, it was established, that molecular weight of GPL was 30 kDa.

With the aim to establish of quaternary structure of GPL, on the next steps of experiments native GPL was denatured in the presence of sodium dodecyl sulphate (SDS) and molecular masses and quantity of its constituent subunits was determined by the method of electrophoresis in polyacrylamide gradient gel (10-25%), using standard calibration protein markers.

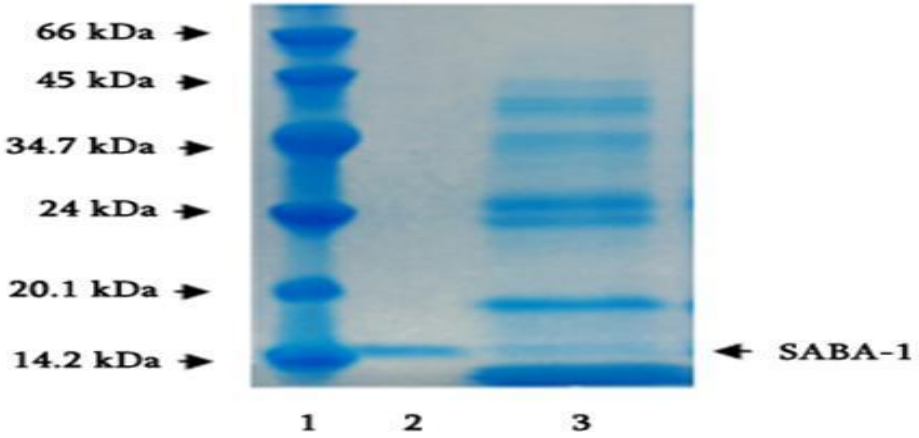

Fig. 4:- GPL electrophoresis in polyacrylamide gradient (10-25%) gel in the presence of the anionic detergent, sodium dodecyl sulfate (SDS). 1.SDS Molecular Weight Markers, Molecular Weight Range 14,000–70,000 Da, gel loading volumes 1-10 µl. (Bovine albumin-66,000; Chicken ovalbumin-45,000; Pepsin, Porcine Stomach Mucosa-34,700; Bovine trypsinogen PMSF treated-24,000; Bovine β-Lactoglobulin-20.1; Egg White lysozyme-14,2); 2. GPL purified by affinity chromatography; 3. GPL containing protein fraction obtained after the gel-filtration on Toyopearl HW-55 column.

As seen from the electrophoregram, presented on Fig 4, the first track was showed the migration profile of markers of the known molecular weight. On the third track.

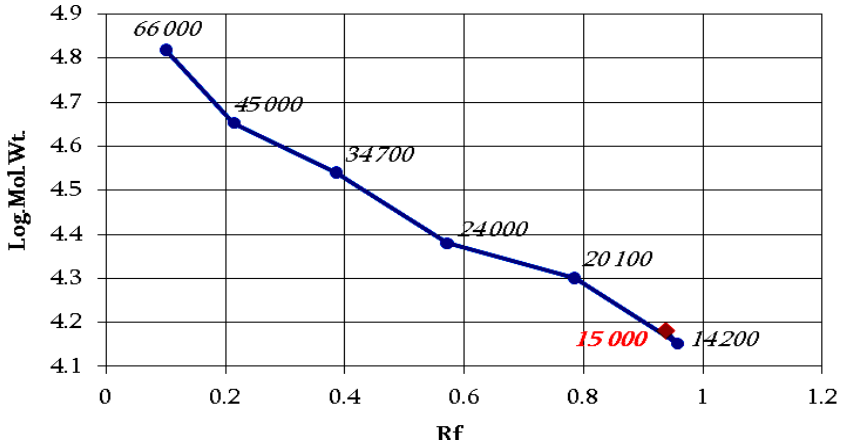

Fig. 5:- Calibration curve with proteins from SIGMA - LMW Range kit 14 –70 kDa, run on polyacrylamide gradient (10-25%) gel in the presence of SDS.

Fig. 5 shows that on SDS-PAGE under reducing conditions, the purified lectin GPL exhibits as a single band, corresponding to a molecular mass of 15 kDa, so. molecular weight of native molecules of GPL corresponds to 30 kDa. Thus it was concluded that GPL consists of two the same subunits and has homodimer quaternary structure.

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IV. ANTICANCER ACTIVITY OF GPL

In order to reveal the nonspecific cytotoxic action of GPL on cancer cells, its was studied the influence of GPL on the short-term cell culture of normal human fibroblasts. According to the obtained results, the 10, 50, 100 μg/ml concentrations of GPL, which are lethal for cancer cells, does not affect normal fibroblast cells.

Data presented in the Table 4 evidence that the same concentrations of GPL had well expressed cytotoxic effects on primary short-term cell cultures, derived directly from the parent tissues of Human skin, lung, ovarian and breast cancer. The results clearly show that growing of the concentration of GPL sharply increases its cytotoxic effect. The maximum cytotoxic effect of GPL towards cancer cells of skin, lung, ovarian and breast cancer is revealed at 100 μg/ml concentration and it attains 68%.

Cancer Types	GPLμg/ml	0	10	50	100	GPL+ α-methyl-mannopyranoside (400 mm)
Skin cancer	AV	0.326	0.283	0.166	0.091	0.320
	NC(%)	100	87	51	28	100
	GI (%)	0	13	49	72	0
Ovarian cancer	AV	0.318	0.218	0.176	0.130	0.315
	NC(%)	100	67	54	40	100
	GI (%)	0	33	46	60	0
Lung cancer	AV	0.328	0.277	0.179	0.121	0.329
	NC(%)	100	85	55	37	100
	GI (%)	0	15	45	63	0
Breast cancer	AV	0.322	0.261	0.192	0.104	0.321
	NC(%)	100	80	59	32	100
	GI (%)	0	20	41	68	0

Table 4:- Ggrowth inhibition effect of GPL on short-term (48 h) primary cancer cell culture derived from human skin, lung, ovarian and breast cancer. AV - Average Value (average value of triplicate measurements of the extinction index); NC (%) - Number of Cells (% of control); GI (%) - Growth Inhibition %.

In the next series of experiments with the aim of revealing molecular mechanisms of cytotoxic action of GPL on cancer cells the effect of α-methyl-mannopyranoside on cytotoxic activity of GPL and GPL mediated cancer cell agglutination was studied.

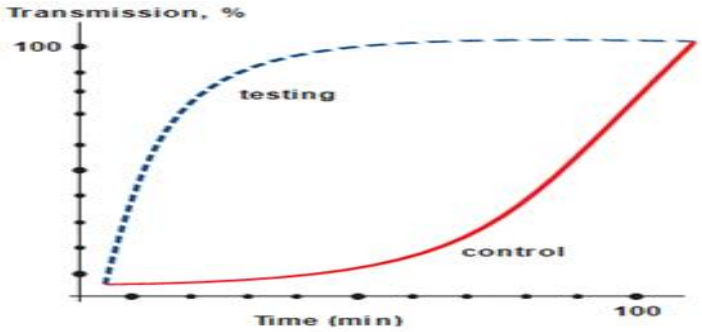

Fig.6:- GPL mediated cancer cells agglutination kinetic assay by the colorimetric method. Control - cancer cells; Testing - cancer cell + GPL

It is impotent to note that GPL cytotoxic activity is fully inhibited by α-methyl- mannopyranoside of the mentioned of all primary in vitro cultures tested tumor cells.

Cancer cell agglutination and growth inhibitory effects of various concentration of GPL were lost at the corresponding level with the lose of mannose-binding activity, indicating that there may be a close link between the agglutinating activity and anti-proliferative property of GPL.

V. CONCLUSION

It was suggested that cytotoxic effect of GPL on cancer cells should be mediated by its specific interaction with α-methylmannopyranoside containing cancer cell membrane surface receptors inducing destruction of cells.

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