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What is the New Tumor Marker for Detection of Different Kidney Tumors? Modern Study to Isolation, Purification, and Characterization of Galactose Binding Lectin from Sera of Patients with Kidney Cancer

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ABSTRACT

The present study was designed to investigate lectins in sera of patients with kidney tumors, in addition to non tumoral kidney disease patients. Fifty five patients of malignant kidney tumors were enrolled in addition to 23 patients of benign kidney tumors, and 18 patients of non tumoral kidney diseases used as control groups, in addition to 46 healthy individuals were also investigated. The age of patients and healthy individuals were 10-90 years. The measurement of total serum proteins revealed significant ($p < 0.001$) decrease in patients of malignant tumors when compared with those of benign, non tumoral diseases, and healthy individuals. The conditions of the hemagglutination assay of serum lectin activity were optimized. They were Tris buffer of 20 mM and pH 7.4, 60 mM $CaCl_2$, 800 µg of defatted serum, 30 ˚C for serum samples, 60 minutes for serum samples, and human blood of group A^+ suspension with 1.4 optical density. The measurement of the specific hemagglutination activity of lectins demonstrated significant ($p < 0.001$) elevation in patients of malignant tumors when compared with those of other patients and healthy individuals. Lectin activity was pointed out to be significantly ($r = 0.767$ at $p < 0.0005$) positively correlated with stage of malignancies. The cutoff value of the specific hemagglutination activity was found to equal 6 SHU for discriminatory malignant kidney tumors. Serum lectins activity were indicated to be inhibited by galactose, mannose, lactose, and N-acetyl galactosamine. Purification of lectin from sera of patients with malignant kidney tumors by affinity chromatography with the use of silver stain revealed galactose binding lectin (GalBL). The purified folds and the yield was 132 with 22.1. The polyacrylamide gel electrophoresis (PAGE) of purified lectin demonstrated one band consisted lectin activity. The approximate molecular weight of GalBL was determined and found to be 98.40. Purified lectin was characterized through the assessment of the capability to agglutinate RBCs, inhibition by EDTA, pH dependency, thermal dependent, and carbohydrate contents. GalBL was observed to be calcium independence lectins. These results suggest that the diagnosis of the specific hemagglutination activity of lectin is a promising biomarker for

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discrimination of malignant kidney tumor patients and the purified lectin could be introduced in the field of biomarkers.

Key Word: lectin, kidney, cancer, galactose, tumor, purification

1 Introduction:

Lectins was first discovered as a highly toxic protein that was isolated from castor tree seeds (Ricinus communis), and named ricin [1], this protein showed the ability to agglutinin erythrocytes [2]. In 1888; Peter Hermann Stillmark, had called this protein as hemagglutinin, or phytoagglutinin, because it was originally found in the extracts of some plants [3- 5]. Kilpatrick [6], Houzelstein et al [7], and Naird et al [8] reported that the lectin was first discovered in snake by Mitchell W in 1860. Mitchell and Stewart described red blood cell agglutination by snake venom in 1883, although this term was not used at that time [6]. Furthermore, in 1886 they published the first study dealed with lectin activity [9, 10]**.** Because of the variation in lectins types, their chemical and physical properties, their roles and applications in the different species were varied too [see reference 11]. Lectins provide way for one molecule to stick to another one without any immunity involved.

They play a wide role in health, but their ability to influence the inflammatory process indicates that they are involved in inflammatory diseases, e.g.: bowel disease, systematic lupus erythematosus, rheumatoid arthritis, and even weight gain [12-15]. Majority of lectin researches have focused on the using of lectins from different sources (other than human) in human medical fields. Somewhat, working with human lectins was surrounded by difficulties, as a result of that, human endogenous lectins studies were, rather, few [16-18].

In human, some lectin genes are expressed constitutively, whereas others are induced by gene activation under specific biological circumstances [19]. Membrane-bound and many soluble lectins are synthesized on ER-bound ribosomes and delivered to their eventual destinations via the ER-Golgi pathway. However, a significant subset of soluble lectins (galectins, heparin-binding growth factors, and some cytokines) are synthesized on free ribosomes and delivered directly to the exterior of the cell by a poorly understood mechanism involving extrusion through the plasma membrane. Some of these lectins can recognize biosynthetic intermediates that occur in the Golgi-ER pathway (e.g., galactosides and high-mannose oligosaccharides) [20].

There are no reliable tumor markers that can be used in the diagnosis, treatment, screening of patients with renal cell carcinoma (RCC) [21], till now general tumor markers in RCCs has been used to assess a cancer's response to treatment and to check for cancer recurrence [22]. Generally, renal cancer is the third most common malignancy of the genitourinary system, and accounts for 3% of adult malignancies globally [23]. The incidence of RCC has been increasing 2 to 4% per year since the 1970s, perhaps related in part to the improvement in and increased use of modern imaging techniques [24]. Limited early warning signs result in late recognition with metastases present in approximately one third of patients at the time of diagnosis, with 210,000 new cases per year and more than 100,000 deaths occurring worldwide annually [25]. The male to female ratio is 1.5:1, and the disease usually occurs in the sixth and seventh decades of life [26]. RCCs are highly vascularized tumors, which may explain the 30-40% prevalence of metastatic disease at initial diagnosis [27], when systemic therapies are then necessary. In

this group of patients, one-year survival rate are ~ 25%, illustrating the limited role of both chemotherapy and radiotherapy in the management of advanced stages of RCC [28]. Advanced RCC responds poorly to systemic therapy and has a 5 year survival rate of less than 10% [23]. Defining the prognosis of RCC is important for both therapeutic decision-making and counseling patients. The prognosis is heavily affected by the extent of disease; the best treatment results can be anticipated in those with minimal tumor spread [29].

2 Materials and methods

Patient and Control Individuals: The present study involved 96 patients (55 cases with malignant kidney tumors, 23 cases with benign kidney tumors, and 18 cases with non tumoral kidney diseases) with the age range 10-80 years, in addition to 46 healthy individuals, at the same age range. The enrolled patients were attended: Al - Sadder Medical City, Al - Ameer Private Hospital, and Al - Ghadeer Private Hospital in Najaf. The diagnosis was confirmed by histopathological examination. Healthy individuals were nonsmokers and they were not subjected to surgical operation.

Serum Samples: Ten milliliters of venous blood samples were collected from patients and the control groups. Samples were allowed to clot at room temperature, centrifuged at 3000 xg for 5 minutes, then sera were collected and stored at -20˚C.

Isolation of Crude Lectins from Serum and Tissue Specimens: Ten milliliters of venous blood samples were collected from patients and the control groups. Samples were allowed to clot at room temperature, centrifuged at 3000 xg for 5 minutes, and then sera were collected and stored at –15˚C.

For isolation of serum crude lectins, 1 volume of serum was mixed with 2.5 volumes of petroleum ether, the mixture was shacked strongly, then, centrifuged at 3000 xg for 5 minutes. The organic phase was neglected and defatted serum was stored at -20˚C to be used for determination of the hemagglutination activity.

Preparation of Standard Ttrypsinized Erythrocyte Suspension for Hemagglutination Test: Human blood group erythrocytes were collected from the local blood bank in Al-Sadder Medical City in Najaf Government in Iraq. Blood was centrifuged at 3000 xg for 5 minutes, the sera were discarded. The erythrocytes were washed with saline solution (5 ml saline: 1 ml packed erythrocytes), then were suspended in phosphate buffer saline solution (pH 7.4), and diluted with the same buffer to give an absorbance of 2 ml at 620 nm.

One part of trypsin solution (1%) was added to 10 parts of the final erythrocytes suspension. the mixture was incubated at 37˚C for 1 hour, and then centrifuged at 5000 xg for 5 minutes. The trypsinized erythrocytes mixture was washed 3 – 5 times with saline solution to remove trypsin traces. Saline solution was added, until the absorbance of the erythrocyte suspension was 1.4 at 620 nm.

Protein Determination: Total proteins in the studied samples were estimated using Bradford method [30], and bovine serum albumin was used as a standard protein.

Determination of Hemagglutination Activity of Crude Serum and Tissue Lectins of Patient and Control Groups: To determine the hemagglutination activity in serum and tissue Lis and Sharon [2] method was used, with essential modifications. The procedure involved three tubes, test (T), blank (B), and control (C). A set of control tubes (2 – 4) were used in each experiment and the assay was carried out as in the

following: The reduction of optical density (ROD) in the test tube (in crude sera and tissues determination) was measured from the following equation:

$$ROD\% = \frac{A_C - A_{T-B}}{A_C} \times 100 \quad (1)$$

Where: A_C: Optical density of cell suspension in the control tube. A_{T-B}: Optical density of cell suspension in the test tube – Optical density of cell suspension in the blank tube. Specific hemagglutination activity unit (SHU) was expressed as hemagglutination units per milligram of protein.

Purification of GalBL: Affinity chromatography technique was applied for the purification of GalBL from patients' with malignant kidney tumors. Preparation of the affinity chromatography column was carried out according to the instructions of Hermanson [31], and Amersham handbook [Amersham Pharmacia Biotech].

Determination of Carbohydrate Content in the Purified GalBL: Dubois method [32] was followed for determination of carbohydrate amount in the purified GalBL. Where glucose was used as a standard sugar.

3 Results and Discussion

Levels of the Specific Hemagglutination Activity in Patients and Control Groups: The optimized conditions of the hemagglutination protocol were used for estimation of individual serum lectin activity in the studied groups. It was expressed as SHU. Figure 1, demonstrates that 52 patients out of the 55 studied patients of malignant kidney tumors have a hemagglutination activity higher than 6 SHU, while those of non tumoral kidney diseases and healthy individuals (except one sample in each group) have less than 6 SHU. Also those of benign kidney tumors were found to have specific activity less than 6 SHU. These results suggest the possibility of using 6 SHU as a cutoff value for the specific hemagglutination activity. A result showed the possibility of using this parameter as a biomarker for discriminating of patients with malignant kidney tumors among those with benign, non tumoral kidney diseases, and healthy individuals.

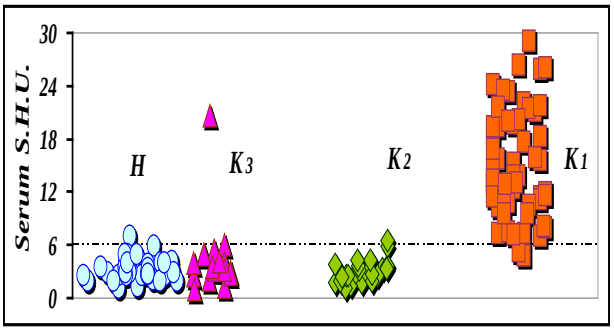

Figure 1: Distribution of the Serum Hemagglutination Activity in Patients of Malignant Kidney Tumors (K_1), Benign Kidney Tumors (K_2), Non Tumoral Kidney Diseases (K_3), and Healthy Individuals (H). The symbol – - – - refer to the cutoff of malignant kidney tumors

The evaluation of the specific hemagglutination activity in the various groups revealed a significant increase ($p < 0.001$) in patients of malignant kidney tumors when compared with those of benign tumors, non tumoral kidney diseases, and healthy individuals. However, non significant variations were obtained when other groups were compared together (table 1). The sensitivity and specificity of serum lectin activity in detection of malignant kidney tumors were 94.54 % and 95.65 % respectively.

Table 1: Serum Specific Hemagglutination Activity Levels in Patients of Malignant Kidney (K_1) and Benign Kidney (K_2) Tumors, Non Tumoral Kidney Diseases (K_3), and Healthy Individuals (HK_1 and HK_2)

Groups	Age (year) Mean ± S.D. Range	SHU Mean ± S.D.	Range	*p*
K_1 (55)	54.93 ± 12.50 32 – 80	14.99 ± 6.21	4.79 – 29.08	0.000** for K_1 vs K_2 0.000** for K_1 vs K_3 0.309 for K_2 vs K_3 0.000** for K_1 vs H_{K1} 0.491 for K_2 vs H_{K2} 0.724 for K_3 vs H_{K2}
K_2 (23)	45.04 ± 15.33 10 – 66	3.04 ± 1.31	1.17 – 6.49	
K_3 (18)	42.39 ± 16.60 12 – 68	4.44 ± 4.27	0.99 – 20.70	
H_{K1} (32)	47.38 ± 10.92 32 – 80	4.27 ± 1.87	1.09 – 9.09	
H_{K2} (43)	39.77 ± 13.77 10 – 66	3.94 ± 1.71	1.09 – 9.09	

*The mean difference is significant at the 0.001 level. **Refers to significant difference between variables.*

Implication of Stages of Malignancy in Serum and Tissue Specific Hemagglutination Activity: In order to verify the changes of the hemagglutination activity with the advancing of malignancy, the patients were subdivided according to the stage of the diseases into stage I, II, III, and IV. From the statistical analysis of the malignant kidney tumors of different stages, a positive correlation was observed between the serum specific hemagglutination activity & the malignant tumor progression ($r = 0.767$ at $p < 0.0005$) (Figure 2).

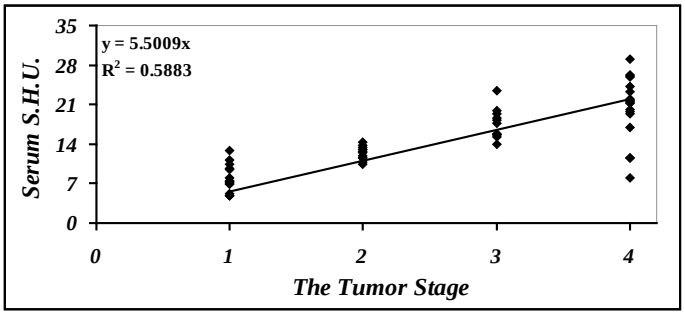

Figure 2: Correlation of Serum Hemagglutination Activity with Stages of Malignant Kidney *Tumors*

The mean levels of specific hemagglutination activity in patients of the four stages of malignant kidney tumors are illustrated in table 2. Significant elevations ($p < 0.001$) of the specific hemagglutination activity were observed when the data of each two stages (except III and IV) were compared.

Table 2: Stage Differences in Serum Specific Hemagglutination Activity of Malignant Kidney Tumor Patients

Subjects	Age (year) Mean± S.D. Range	SHU Mean± S.D.	Range	*p*
Stage I (14)	*49.07 ± 11.94 32 – 74*	*8.03 ± 2.40*	*4.79 – 12.80*	*0.000** For (1, 2, 3, 4, and 5) 0.011 for (6)*
Stage П (12)	*55.67 ± 13.85 34 – 79*	*12.40 ± 1.21*	*10.37 – 14.42*	
Stage Ш (11)	*53.73 ± 9.71 43 – 75*	*17.58 ± 2.73*	*13.87 – 23.47*	
Stage IV (18)	*59.72 ± 12.40 41 – 80*	*20.55 ± 5.57*	*7.97 – 29.08*	

*The mean difference is significant at the 0.001 level. **Refers to significance between the variables. 1)Stage I vs. Stage II, 2) Stage I vs. Stage III, 3) Stage I vs. Stage IV, 4) Stage II vs. Stage III, 5) Stage II vs. Stage IV 6) Stage III vs. Stage IV*

Gender Involvement in Kidney Lectins Hemagglutination Activity Changes: The effect of gender on the kidney specific hemagglutination activity levels in patients of cancerous tumors, benign tumors, and non tumoral kidney subgroups was evaluated. **Student's *t-test*** failed to exhibit significant changes among male and female patients (Table 3).

Table 3: Gender Differences of Serum Specific Hemagglutination Activity in Tumoral and non Tumoral Kidney Disease Patients and Healthy Individuals

Type	Gender	Age (year) Mean ± S.D. Range	SHU Mean ± S.D.	Range	*p*
K_1 (55)	M (36)	*57.31 ± 13.69 32 – 80*	*15.48 ± 6.94*	*4.79 – 29.08*	*0.259*
	F (19)	*50.79 ± 9.19 37 – 65*	*14.08 ± 4.55*	*7.97 – 21.69*	
K_2 (23)	M (14)	*43.93± 16.73 10 – 66*	*2.40 ± 0.77*	*1.17 – 3.59*	*0.377*
	F (9)	*47.44 ± 12.28 25 – 62*	*4.04± 1.38*	*2.45 – 6.49*	
K_3 (18)	M (11)	*47.36 ± 11.33 27 – 62*	*3.95 ± 1.23*	*2.05 – 6.00*	*0.550*
	F (7)	*34.57 ± 21.22 12 – 68*	*5.21 ± 6.92*	*0.99– 20.70*	
H (46)	M (21)	*44.24 ± 9.57 10 -81*	*4.69 ± 2.08*	*1.09– 9.09*	*0.432*
	F (25)	*44.88 ± 17.10 11– 87*	*3.53 ± 1.14*	*1.09 – 6.13*	

K_1:Malignant Kidney Tumor Patient group, K_2: Benign Kidney Tumor Patient group, K_3: Non Tumoral Kidney Patients, and H: total healthy individuals. M: Male, F: Female. The mean difference is significant at 0.001 level

Previously, the source of increased serum lectins in cancer patient was reported to be unclear [33]. The significant positive correlations of serum and tissue lectins of patients with malignant tumors reported here suggest presence of a direct relationship between their lectins levels which means that the malignant tumors are the sources of lectin present in the sera of the malignant patients [data not shown].

In the present study, removal of the tumors, decreased serum hemagglutination activity, thus tumor tissues are most likely to produce and secrete lectins in sera. The agglutination test of cancerous tissues showed that lectin was found not only on malignant cells but also in macrophages and stromal cells (mainly fibroblasts) near cancer focus, and the stromal cells immediately adjacent to cancer nests was found to have higher levels of the hemagglutination activity in comparison to cells far from the nests. These results suggest that circulating lectins are generated not only by tumor cells but also from peritumoral inflammatory cells and stromal cells. Different modalities have been proposed to explain how lectins might be involved in the metastatic process: (1) Lectins act as a bridge molecule enhancing the adhesive interactions between tumor cells and the extracellular matrix. (2) Several lectins are able to mediate homotypic cell-cell adhesion through interaction with complementary glycoproteins depending on the hypothesis that lectins are involved in the formation of tumor emboli and dissemination of tumor cells in the circulation. (3) Lectins are able to protect the malignant cell against apoptosis induced by the loss of cell anchorage. The expression of lectins in tumor cells may provide a critical determinant for cell survival of disseminating cancer cells in the circulation during metastasis [33].

In patients with benign tumors serum hemagglutination activity was found to remain within values of healthy individuals, this is due to the differences of benign from malignant tumors. In contrast to the malignant cells, benign tumor cells are under control. On the other hand, during benign tumor formation, several lectins, which extend normally on the cell surface, are degraded and others are built, these processes are contributed in keeping lectin concentration balance [34].

Various lectins from different species are studied for evaluation of their roles in cancer treatment, and therapy. Preliminary findings suggest that some lectins, but not all; can detect alterations of malignant cells as well as reduce the cancer cell tumorigenicity, thus may have benefits for the immune status of the patients. A lectin from Viscum album (mistletoe) for instance is known to increase the reactivity of the lymphocytes of tumor-bearing mice to the mitogens *in vitro*, thus indicating its immune stimulating effects for cancer-immunosuppressed lymphocytes. It also inhibits the protein synthesis in various malignant cell lines. Similarly, because of the cytostatic/apoptotic and immunomodulatory effects of the mistletoe lectin, the extracts are often applied in the treatment of tumor bearing patient [20].

Purification of Serum Human Gal BL: Hydrophobic affinity chromatography was used for isolation and purification of galactose binding lectin from sera of patients with malignant kidney tumors, benign kidney tumors, non tumoral kidney diseases, and healthy individuals. The purification protocol was carried out by using sepharose 6B column activated with bis-oxirane (1, 4 – Butanediol diglycidyl ether) ($C_{10}H_{18}O_4$). The chromatograms of the purified lectins was demonstrated in figures 3. The purification folds and the yield percentage of GalBL was 132 with 22.1 % (table 4).

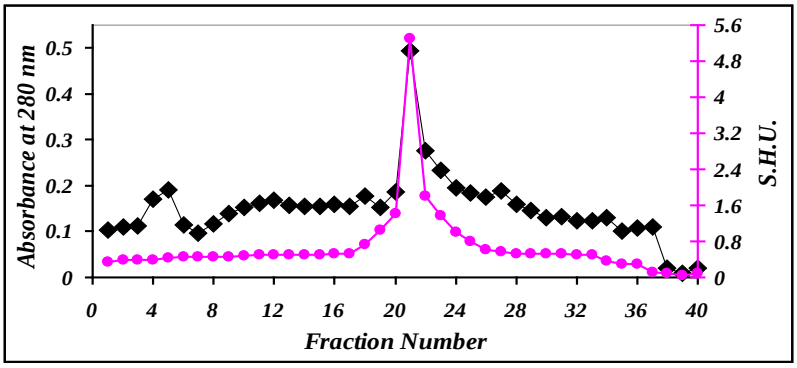

Figure 3: Affinity Chromatogram of Malignant Galactose Binding Lectin (GalBL) using Sepharose 6B // Galactose Column (1.6×1.3) at Flow Rate 30ml / hour. The volume of each fraction was 1ml.Tris Buffer 20 mM and 7.4 pH was used as a Washing Solution. The Elution step was carried out using a Tris Buffer (20 mM, pH 7.4) contained 30 mM Galactose.

Table 4: Results of the Purification Protocol of Lectins from Sera of Patients with Malignant Kidney Tumors

Purification step	Total volume (ml)	Total protein (mg)	Total activity (HU)	SHU (HA/µg of protein)	Purification (fold)	Yield %
MKT Serum	3	45	44.325	0.985	1	100
GalBL	5	0.0750	9.783	130.440	132	22.1

MKT: Malignant Kidney Tumor Serum; GalBL: Galactose Binding Lectin

The purification protocol was applied for the analysis of sera from patients with benign kidney tumors and non tumoral kidney diseases, in addition to healthy individuals, sera of the three groups of enrolled individuals failed to appear same malignant sample results.

In various lectin studies, “conventional” procedures including salt-induced crystallization, ethanol precipitation, ion exchange chromatography, and gel filtration, or affinity chromatography have been used in isolation and purification [15,35- 37]. The former methods depend on physicochemical properties of proteins while affinity chromatography depends on specific interactions between lectins and a carbohydrate attached to an inert matrix. Although affinity chromatography is a highly specific procedure, some carbohydrate matrices used for affinity chromatography can adsorb not only the lectin but also glycosidases capable of hydrolyzing the sugar structures to which the lectin may bind. Contamination by glycosidases could greatly affect the activity of a lectin preparation [31].

The comparison of the chromatographic pattern of patients with malignant kidney tumors, benign kidney tumors, and non tumoral kidney diseases as well as the healthy individuals exhibits that GalBL could be isolated and purified successfully from sera of patients with malignant kidney tumors, and malignancy of kidney is associated with overexpression of GalBL.

Many laboratories have made considerable efforts to identify a ‘poor prognosis signature’ of tumor- and metastasis-associated genes and to predict the clinical outcome of neoplastic disease. Changes in glycosylation have been shown to strongly associate with the development of cancer and metastasis.

This differential glycosylation of cancerous and healthy tissues is often restricted to altered glycan expression of tumor cells or their secreted glycoproteins [38,39]. In many cases, the structural changes have been further correlated with changes in the activity of one or more glycosyltransferases during the process of transformation from normal to tumor cells. Changes in glycosylation could result in loss of cell adhesion, an event associated with increased cell invasiveness of primary tumors to distant sites [39,40].

Analysis of Serum Crude and Purified Lectins of Patients with Tumoral and Non Tumoral Kidney Diseases and Healthy Individuals: Crude and purified serum proteins were analyzed by polyacrylamide gel and stained with Periodic acid and Silver stains. Periodic acid stain of crude pooled sera of patients and the control group revealed qualitatively incompatible electrophoretic pattern in particular for patients with malignant tumors (figure 4).

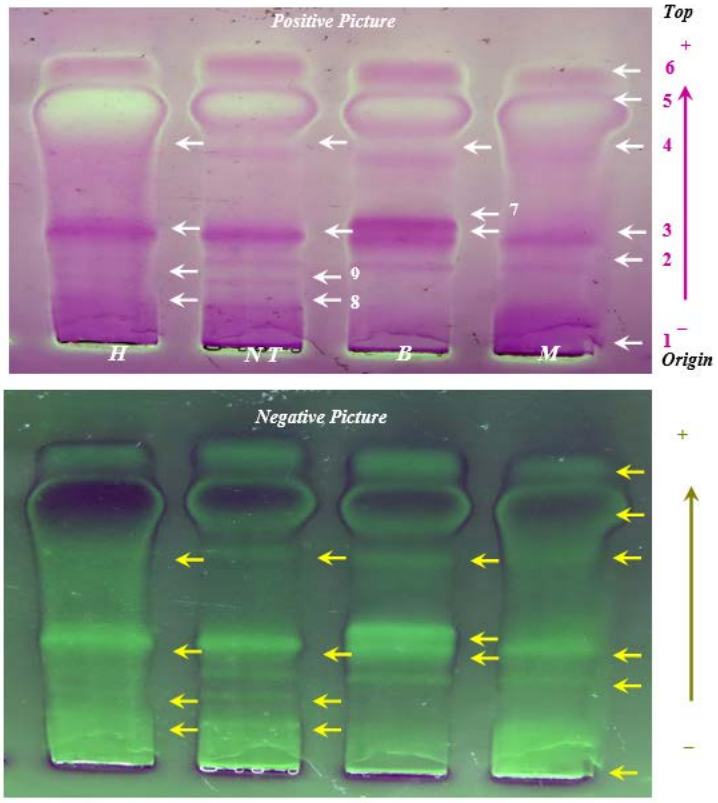

Figure 4: Electrogram of Glycoproteins profile using Conventional Polyacrylamide Gel Electrophoresis (PAGE) 7.5%. Tris - glycin buffer (0.075 M, pH 8.9) was used as the electrode buffer. Prelctrophoresis conditions were 50 mA as a constant current for 30 minutes, with voltage of 15 v/cm, and at 4 ºC. Electrophoresis was carried out for10 minutes at 20 mA. The process was continued for 3.5 hours at 4 ºC by using 40 mA as a constant current and voltage of 15 v/cm. The gel was stained for protein with periodic acid-Schiff. The crude samples that applied were: M: Malignant Kidney Tumors Sera, B: Benign Kidney Tumors Sera, NT: Non Tumoral Kidney Affections Sera, H: Healthy Individuals Sera

The additional bands that appear in glycoprotein profile, particularly in the globulins region due to the changes in cell surface glycoproteins during malignancy, and (or) as a result of synthesis of the acute phase proteins during inflammation processes [40].

Figure 5: Conventional Polyacrylamide Gel Electrophoresis (PAGE) 7.5% for Proteins. Tris - glycin buffer (0.075 M, pH 8.9) used as the electrode buffer. Prelctrophoresis conditions were 50 mA as a constant current for 30 minutes, with voltage of 15 v/cm, and at 4ºC. Electrophoresis was carried out for10 minutes at 20mA, then the process was continued for 3.5 hours at 4ºC by using 40 mA as a constant current and voltage of 15 v/cm. The gel was stained for protein with silver stain. The crude and purified samples that applied were: *Crude Malignant sera sample A: purified GalBL

The approximate molecular weights of purified lectins were determined using conventional PAGE. Five standard proteins with known molecular weighs were used to construct the standard curve as shown in table 5.

Table 5: Standard Proteins Used for the Determination of Purified Lectin Molecular Weights

Standard Proteins	Molecular Weigh (kD)	Number
Lysozyme	13.6	1
Chemotrypsinogene	25	2
Ovalbumin	47	3
Bovine Serum Albumin	67	4
Lactate Dehydrogenase	140	5

The estimated molecular weight of GalBL was 98.40.

The molecular weight of GalBL from calf heart and lung was determined to be approximately 17 kD, and the predominant molecular species had a molecular weight of 9 kD [41]. The molecular weight of the undenatured GalBL purified from human placenta by gel filtration was found to be 26·9 kD. When SDS electrophoresis was applied for this lectin, two subunits with the molecular weight approximate to 13.4 kD for each have been identified [42]. The native molecular mass of galactose binding lectin from seeds of Erythrina speciosa detected by hydrodynamic light scattering was 58 kD and when examined by mass spectroscopy and SDS–PAGE was found to be composed of two identical subunits of molecular mass of

27.6 kD [43]. The purified GalBL from the Latex of Synadenium carinatum lectin appeared to be a glycoprotein with apparent molecular weight of 120 - 130 kD made up of polypeptide chains of 28 and 30 kD [44]. In another study, GalBL purified from pinto beans with a molecular mass of this homodimeric lectin of 62 kD and that of each of its subunits was 31 kD [45].

Characterization of Purified Lectins: To examine the sensitivity of purified lectins in the agglutination reactions, blood of various groups were included in the experiment. Purified GalBL from sera of patients with malignant tumor kidney from sera of patients with malignant kidney tumors was showed its ability to agglutinate RBCs, and the highest agglutination activity was recorded with A^+ blood group. These findings could be explained by differences in glycosylation of the surface proteins of red blood cells [46].

The hemagglutination process was carried out for the purified GalBL in the presence of EDTA. GalBL did not lost the hemagglutination activity, even at concentration of 9 mM of EDTA (which was recorded as the highest concentration of EDTA to complete the inhibition of lectin hemagglutination to crude sera of human lectins). This result clearly indicate that Ca^{2+} is not crucial for the expression of the hemagglutination activity of this lectin, thus; GalBL should be classified as independent calcium lectin type.

The effect of pH on the activity of the purified lectin was investigated. Figure 6 points out that maximal lectin activity was achieved at pH 7.4, while the purified lectin was sensitive to acidic (pH 3) and to basic (pH 12) conditions, under which the activities were completely lost.

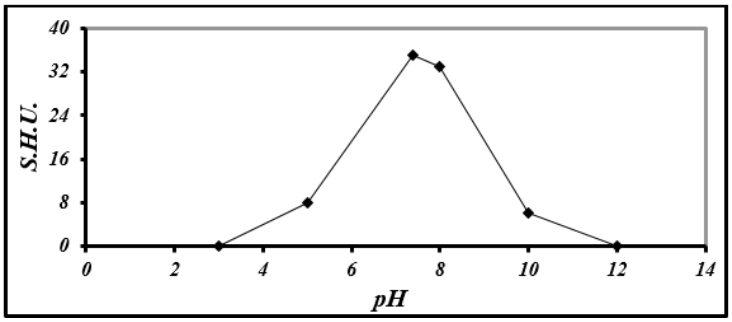

Figure 6: Effect of pH on Purified GalBL Hemagglutination Activity

The conformational change of purified lectin may occur by weakening hydrogen bonds between water and polar groups. Alternatively, the excessive pH may directly affect the interaction between amino acid residues within the protein monomer and facilitate conformational change upon the binding of specific carbohydrates, leading to hydrophobic interaction between the lectin molecules. Moreover, changes in the surface hydrophobicity of the purified lectins may be one of the possible factors affecting the increased irreversible binding to the erythrocyte membrane. According to that, the close relationship between the hydrophobicity enhancement and the oligomerization of purified lectins upon the binding of carbohydrates. Therefore, it seems reasonable to suppose that the hydrophobicity enhancement and the oligomerization of purified lectin reflect structural changes of the purified lectin, which occur during interaction with the erythrocyte membrane.

To explore the effect of temperature on the hemagglutination activity of purified lectin, it was incubated at various temperatures (0°C, 30°C, 40°C, 50°C, 60°C, 80°C, and 100°C) for 1 hour; the mixtures were

cooled until room temperature. The hemagglutination activity was carried out at 30°C (as a optimum temperature to crude lectin hemagglutination). Thermal denaturation results revealed that purified lectin remained stable below 40ºC for 1 hour with no loss of hemagglutination activity, while; it loss about 40% of its hemagglutination activity at 50 ºC. Lectin activity disappeared when the denaturation was carried out at more than 60 ºC (figure 7).

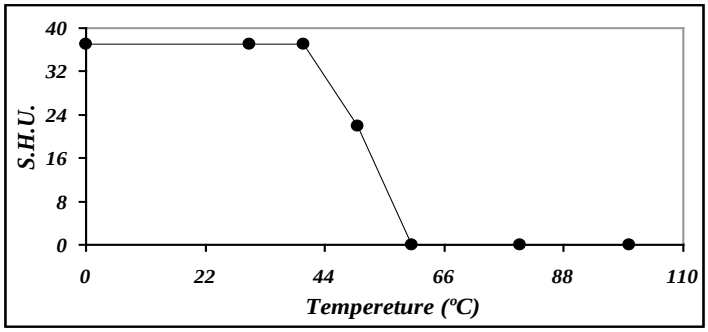

Figure 7: Thermal denaturation of Purified Lectin Hemagglutination Activity

Both 30 and 37 ˚C seem to be more suitable among the examined temperatures for the agglutination process of purified GalBL.

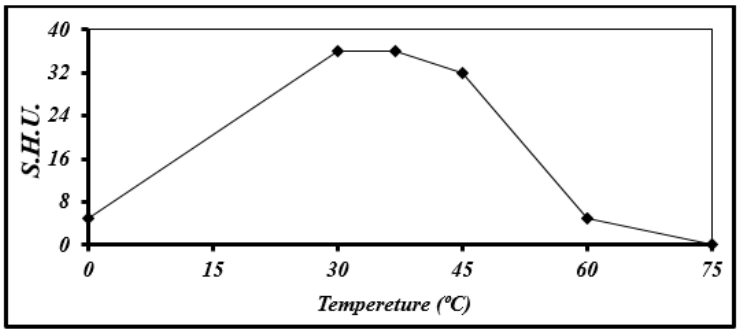

Figure 8: Temperature Effect on the Purified GalBL Hemagglutination Activity

The gradual increase in the purified lectins activity, when the agglutination reaction temperature rises until it reaches its optimum temperature, may be due to the elevation of the kinetic energy for the electrons that leads to weakening or disruption of bonds among the chains of purified lectins, which increase the collision between the interacting molecules, while the observed decrease in this activity when the temperature is more than 37 ˚C may be due to the denature of the protein molecules.

Total carbohydrate contents were estimated by phenol-sulphuric acid method [32] with D-glucose as standard sugar. Carbohydrate was found to compose about 10.8% of GalBL. The results of present study was near to other studies that isolated GalBL from different species [43,47-49].

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