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Cytotoxic effect of the purified lectin from locally Isolate *Acinetobacter baumannii* on Hep-2 tumor cell line

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Abstract

Lectins are glycoproteins of non-immune origin which have a wide range of application in medical field. In this study, a novel strain, *Acinetobacter baumannii* S_4 gave the highest production level of lectin by microscopic glass slide and microtiter plate methods and found that lectin agglutinated non-specifically red blood cells of human type A^+, B^+, AB^+ and O^+ also the blood group O^+ was the best among the other blood groups. Purification of the lectin was achieved by35% saturation ammonium sulfate followed by affinity ion-exchange chromatography on DEAE -cellulose column and gel filtration chromatography Sephadex G-75 column with a yield of 36% and a purification fold of 4.66.This study showed that the purified lectin has a cytotoxic effects on cancer cell (Hep-2) as could be seen from their effects on inhibition percentage and the significant differences ($p<0.05$) which was observed by increasing the inhibition percentage as the concentration and time were increased. The higher level of inhibition(64%) was obtained at concentration56.25µg/ml after 72hour of exposure and when the concentration was increased more than56.25µg/ml, the inhibition rate was decreased.

Introduction

Acinetobacter species are aerobic gram-negative cocco-bacilli, ubiquitous, free living organisms(1). The most predominant position of infection is the respiratory tract. However, infection in the blood, urinary tract and other sites has also been described(2). *Acinetobacter* prefer moist environment and therefore it was isolated from from soil, water, food and sewage. They are usually considered to be opportunistic pathogens, and in the recent years have been reported to cause a number of outbreaks of nosocomial infections in hospitalized patients like septicaemia, pneumonia, wound sepsis, endocarditis, meningitis and urinary tract infection (UTI)(3). In the hospital environment, *Acinetobacter baumannii* can colonize the respiratory, urinary, gastrointestinal tract and wounds of the patients and can cause infections in burn, trauma, mechanically ventilated and immunocompromised patients. It shows a special predilection for the ICU(2).The colonization of *Acinetobacter baumannii* to different host tissues determined with adhesion factors .The bacterial strain has lost an adhesions factors loss their ability to survive, these virulence factor called lectins that can recognize the glycoconjugates that were found on other cells (4).

Lectin was originally called hemagglutinin or agglutinin due to its ability to agglutinate of human as well as animal erythrocytes. Lectins and hemagglutinins are a heterogeneous group of proteins or glycoproteins of non-immune origin which have at least one non-catalytic domain that exhibits reversible binding to specific monosaccharides or oligosaccharides(5). These proteins are ubiquitous in nature, and occur in animals, plants, bacteria, viruses, and fungi(6). Lectins are used as tools for identifying and mapping sugars on cell surfaces because of its agglutinating property and their ability to bind glycoconjugates(7). As a result for their chemical properties, they have become a useful tool in several fields such as immunology, cell biology, molecular biology, membrane structure, pharmacology, cancer research, clinical chemistry and genetic engineering. So that the aim of present study to screen the lectin productivity by *Acinetobacter baumannii* in addition to purification and investigation the cytotoxic effects of lectin on viability of cancer cell line(Hep-2).

Material And Methods

Sample collection

Forty samples of sputum were collected from different patients suffering from respiratory tract infections.

Bacteriological analysis

The collected samples were transported to the laboratory. One loopfull of each samples was streaked on blood agar and MacConkeys agar, then incubated at 37°C for 18-24 h. The pure culture isolates were identified to species level by doing some biochemical characterization such as a negative oxidase test, positive catalase test inability to motile coccobacilli(8). *Acinetobacter baumannii* isolate was confirmed by using API 20E biochemical kit and the Vitek 2 system by using Vitek GNI card (bio Mérieux, France) according to the manufacturer's instructions.

Lectin production

1-Semi-quantitative analysis

The semi-quantitative screening was done on microscopic glass slide as following: twenty- five µl of bacterial suspension at dilution 10^{-9} was mixed with 25 µl of 0.02M Phosphate Buffer Saline (PBS) pH 7.2 on glass slide, then 25 µl of blood suspension for human erythrocytes types A^+, B^+, AB^+ and O^+ at a concentration of 3%, blending the mixture well by wooden chopsticks and then moved the glass slide and gently examined

near the light source to note agglutination. An agglutination within 5 minutes refer to positive result. The control was PBS with blood instead of bacterial suspension(9).

2- Quantitative hemagglutination assay

The quantitative screening was done in microtiter plate as following: a serial two-fold dilution of bacterial suspension or lectin solution (50 µl) in microtiter U-plates with 0.02M Phosphate Buffer Saline (PBS) pH 7.2 was mixed with the same volume of a 3% suspension of human erythrocytes in the same buffer and incubated at 37°C for 2 hours. The activity was expressed as hemagglutination units (H.U.). One H. U. was defined as the inverse of the highest dilution still capable of causing agglutination(10).

Estimation of protein content

The protein content of lectin was determined by using the method of (11) and using bovine serum albumin as a standard.

Extraction of lectin

The bacterial isolate was grown on Colonization Factors Antigens medium (CFA) composed of the following (g/L): gasamino acid,10;Yeast extract,1.5;$MgSO_4$,0.05; $MnCl_2$,0.005 at 37°C for 24 hour, then the cells was harvested by centrifugation at 8000rpm for 30 min, washed twice and re-suspended in 0.02M Phosphate Buffer Saline (PBS) pH 7.2. Cells were disrupted by glass beads for 50 min at 4°C using the vortex. Residual whole cells and cell membrane fragments were removed by centrifugation 8000 rpm for 20 min. The hemagglutination activity of lectin and protein concentration were measured for the resulting crude cell extract.

Purification of lectin

The crude cell extract was fractionated with ammonium sulfate at concentrations 20-60% saturation and the precipitate obtained after centrifugation at 8000 rpm for 30 min was suspended in 0.02M Phosphate Buffer Saline (PBS) pH 7.0 and the hemagglutination activity and protein concentration were measured.

The dialysed protein was applied to a DEAE -cellulose column (2.5×20cm) previously equilibrated with the same buffer. The protein was washed with the same buffer and eluted with a salt gradient containing 0.1–0.5M NaCl. The hemagglutination activity for each fraction was assayed as described above. The fractions that revealed significant peak of activity were mixed together and applied to a Sephadex G-75 column (2×80cm) previously equilibrated with the same buffer. Elution was performed with the same buffer, the fractions that revealed the protein and hemagglutination activity in the same peak were mixed and used for further study.

Cell line growth and cytotoxicity assay

The cytotoxic effect was tested for purified lectin on growth of cancer cell line Hep-2 (Human larynx epidermoid carcinoma) and Rat embryo fibroblast (REF) which were provided by the center Biotechnology research center of Al-Nahraine University. All solutions were prepared at the same and cultured tissues were studied *in vitro* under optimum conditions. The growth media used in tissue culture technique used in tissue culture technique was MEM (Minimum Essential Media) which contains fetal calf serum (10%) to form a confluent monolyer, then subcultured to discard the previous growth medium and the cells washed with sterilized phosphate buffer solution (PBS) then 2-3 ml of trypsin versene solution was added for 3-5 min with stirring. The trypsin- versene solution was discarded and the cells were incubated at 37ºC until the separation of the cells from the ground flask.

In cytotxicity assay, the cell line Hep-2 was treated with lectin using four concentrations (28.12,56.25, 112.5 and 225µg/ml) .Immediately 25 ml of trypsin-versene solutions was added into culture bottle and 20 ml of culture medium which contains 10% of serum to provide the suspended cells, mixed very well and 0.2 ml was added to each microtiter. The plates were incubated at 37ºC for 24 hour to form monolayer, then the previous culture medium which presents in to the plates was discarded . 0.2 ml of the lectin under study was added and repeated as negative control (cancer cell line Hep-2 with buffer solutions) and incubated at 37ºC for 24,48 and 72 hour. The culture medium was discarded from microtiter plates, then 0.2 ml of crystal violet solution was added to wells and the plates were incubated for 20 min at 37ºC. The plates were washed gently with distilled water and left to dry. At the end of assay the plates were examined by ELISA reader at 492nm. Only viable cells able to take the stain while the dead cells were not. The inhibition percentage was measured according to(12) and as follows:

Inhibition percentage % =((Absorbance of negative control – Absorbance of test)/ Absorbance of negative control)× 100

Statistical analysis

The experiments data were analyzed using statistical software SPSS version 16, significant differences between control and sample means were assessed using student´s T-test and P values ≤ 0.05 were considered significant.

Results And Discussion

Isolation of *Acinetobacter baumannii*

Five (12.5%) *Acinetobacter baumannii* isolates were obtained out of 40 samples of sputum. *Acinetobacter baumannii* was isolated from respiratory tract infections (sputum) in high isolation rate (13). A low number of

Acinetobacter belonging to other groups such as blood and urine, this revealed that they also related with different disease, but less frequently than isolates from sputum(14).Most nosocomial infections such as urinary tract, respiratory tract, wound and blood infections are related with *Acinetobacter* spp.(15). (16) reported that among all nosocomial bacterial isolates, *Acinetobacter* sp. was found at isolation rate of 18%. The ability to colonize the animate equipments in the hospital and persistent on these surfaces for several months(15). In contrast, (17) found that the isolation percentage of *Acinetobacter baumannii* about 48% from sputum specimens.

lectin production

1-Semi-quantitative analysis

The results revealed that human erythrocytes like A^+, B^+,AB^+ and O^+ gave hemagglutination activities with all *Acinetobacter baumannii* isolates and the isolate *Acinetobacter baumannii* S_2 showed higher hemagglutination level (+ + + + +) with O^+ blood group (table-1). Blood group O^+ found the best among the other blood groups, then B^+ blood group,in addition, blood groups A^+ and AB^+ gave hemagglutination in the lowest level.

2- Quantitative analysis

The hemagglutination activities in microtiter plate were revealed in all bacterial isolates and *Acinetobacter baumannii* S_4 showed higher hemagglutination value against O^+ blood group then B^+ blood group in the second rank, in contrast, while the remaining blood groups showed lower values(table-1),therefore; these results demonstrate that hemagglutination activity was not blood-type specific. The O^+ blood group revealed higher ability of hemagglutination with bacterial isolates of *Acinetobacter baumannii* (18), the pili that found in *Acinetobacter baumannii* have an ability to hemagglutinate the blood group type O^+(19). Lectins produced by ***Artocarpus camansi*** **and** *Mycobacterium smegmatis* showed high ability to agglutinate different human erythrocytes besides to animals erythrocytes(20,21).

Table-1: Hemagglutination activity for *Acinetobacter baumannii* isolates by microscopic glass slide and microtiter plate methods

Isolate No.	Degree of hemagglutination for Human erythrocytes				Titer of hemagglutination for Human RBCs				blood group type O^+	
	A^+	B^+	AB^+	O^+	A^+	B^+	AB^+	O^+	Conc. of Protein (mg/ml)	Specific activity (U/ml)
AS_1	+ +	++ +	+	+ + +	16	32	8	32	5.23	6.11
AS_2	+ +	+ + +	+	++ + + +	8	32	8	64	6.65	9.62
AS_3	+ +	+ + +	+ +	+ + + +	16	32	16	64	6.33	10.11
AS_4	+ + +	+ + + +	+ +	+ + + + +	32	64	16	128	9.12	14.03
AS_5	+ +	+ +	+	+ + + +	8	16	8	64	5.26	12.16

Extraction and purification of lectin

The glass beads was used to extract of lectin and found that the hemagglutination activity raised to 512U/ml with specific activity(162.53 U/mg). The disruption with glass beads is preferable method when proteins very sensitive and easily lose their activity and when a high protein concentration is needed. Small beads are the most effective for bacterial disruption. Typically 100 µm zirconium or silica beads are the most effective for disrupting bacteria and provision of high yields of analytes. If proteins or cellular components are sought the bead beating and sonication are both proven alternative techniques to disrupt bacteria(22).

The purification procedure of lectin included three steps started with ammonium sulfate saturation from 20 to 60%, followed by ion exchange chromatography by DEAE-cellulose column and gel filtration chromatography by sephadex G-75 column. An ammonium sulfate fractionation revealed that 35% saturation led to increase the specific activity to 208.13 U/mg. Ammonium sulfate was chosen as precipitating agent in this study due to its high solubility in water and produces high ionic strength. Increase in ionic strength decreases the protein solubility(23). Desalting was done by using the dialysis with phosphate buffer saline and the dialysis was performed in the cold condition to prevent possible denaturation of the protein. this step led to remove ammonium sulfate from the sample. After dialysis step, the sample was applied to DEAE -cellulose column. A gradient concentrations of NaCl from 0.1 to 0.5M were used in elution process and led to appear four peaks of proteins and only third peak showed hemagglutination activity(figure-1) in this step 44% the yield of lectin and 3.18 a purification fold. The collected fractions was loaded on sephadex G-75. The eluted fractions appeared two different peaks of protein and the hemagglutination activity was found in the second protein peak (figure-2). The lectin was purified with a yield of 36% and 4.66 a purification fold(table-2).

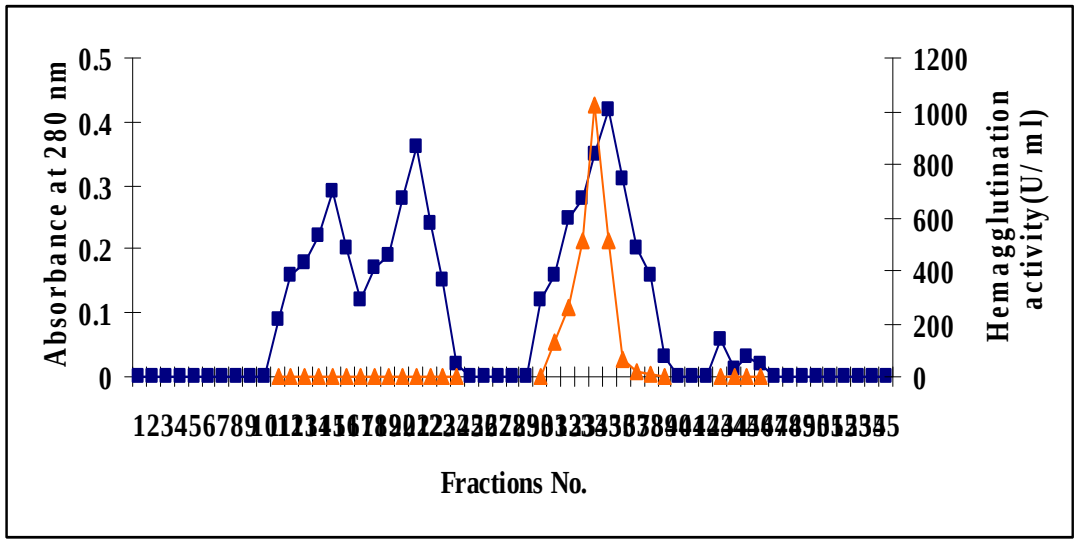

Figure (1): Ion exchange chromatography on DEAE -cellulose column for Purification of *Acinetobacter baumannii* lectin. (▲)lectin activity, (■)concentration of protein.

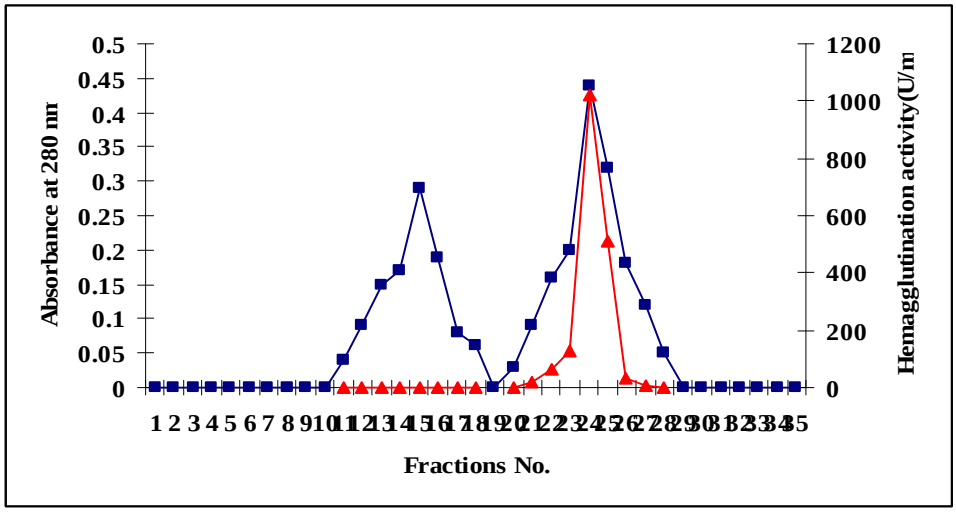

Figure (2): Gel filtration chromatography on sephadex G-75 column on sephadex G-75 for Purification of *Acinetobacter baumannii* lectin. (▲)lectin activity, (■)concentration of protein.

Table (2) : Steps of lectin purification from *Acinetobacter baumannii*.

Purification step	Size (ml)	Hemagglutination activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/ mg)	Total activity	Purification fold	Yield (%)
Crude extract	50	512	3.15	162.53	25600	1	100
$(NH_4)_2SO_4$precipitation	24	512	2.46	208.13	12288	1.28	48
DEAE -Sephadex	11	1024	1.98	517.17	11264	3.18	44
Sephadex G-75	9	1024	1.35	758.51	9216	4.66	36

The cholera lectin was purified from *Vibrio cholerae* strain CA401 with a yield of 6% by ammonium sulfate precipitation followed by gel filtration, and isoelectric focusing (24). a novel lectin from the seeds of Sophora alopecuroides, was purified by ion-exchange chromatography on (DEAE)- and (CM)-Sepharose columns, followed by gel filtration on a Sephadex 75 10/300 GL(25).

Cell line growth and cytotoxicity assay

The *in vitro* growth inhibitory effects of purified lectin was assayed for four concentrations (28.12,56.25, 112.5 and 225µg/ml) against tumor cell line (Hep-2) and normal cell line (REF). The results revealed a significant ($P<0.05$) increasing inhibition rate of Hep-2 cultured cells as compared to control cultures, ranging from 10% to 20%, 15% to 20% and 33-64% at concentrations ranged from 28.12 to 56.25µg/ml at 24h ,48h and 72h of exposure time, respectively. When the concentration was increased more than56.25µg/ml, the inhibition

rate was decreased to 15,22 and45% at concentration 225µg/ml at 24,48 and 72h of exposure time, respectively as shown in figure(3).The highest rate of inhibition was 64% at concentration 56.25µg/ml after 72hour of exposure. According to these results we can conclude that the inhibition rate was concentration and time dependent on cancer cell (Hep-2). This can be attributed to sensitivity of Hep-2 cell and may be due to the variation in the cytotoxic activity of lectin according to different time of exposure.

Lectins lead to apoptosis in different cancer cell lines by the apoptotic factors that work in different apoptotic pathways including activation of different caspases such as caspase-3 that plays a central role in apoptosis where It interacts with caspase-8 and caspase-9. Apoptosis can be mediated by death receptors initiated by lectins. FAS receptor is the receptor with which lectins often interact. The sequestration of cytochrome c in mitochondria was interrupted. Cytochrome c release was observed and finally, mitochondrial membrane depolarization was detected(26,27). The ability of inhibition or enhancing apoptosis by lectin depends on several factors such as; protein concentration and concerted action of protein and cell type(28).The differences in Hep-2 response toward different treatments might indicate the presence or absence of specific cellular receptors in each type of cell lines, making the cells interacts at same concentration in different manners. Moreover the metabolic pathways in response to each treatment differed from one line to another(29). *Sophora alopecuroides* lectin (SAL) had cytotoxic activity for human cervical cancer cells (HeLa)(anticancer). The lectin purified Schizophyllum commune was shown to be a glycoprotein with cytotoxic activity against human epidermoid carcinoma (KB) and human cervical carcinoma (HeLa)(30).

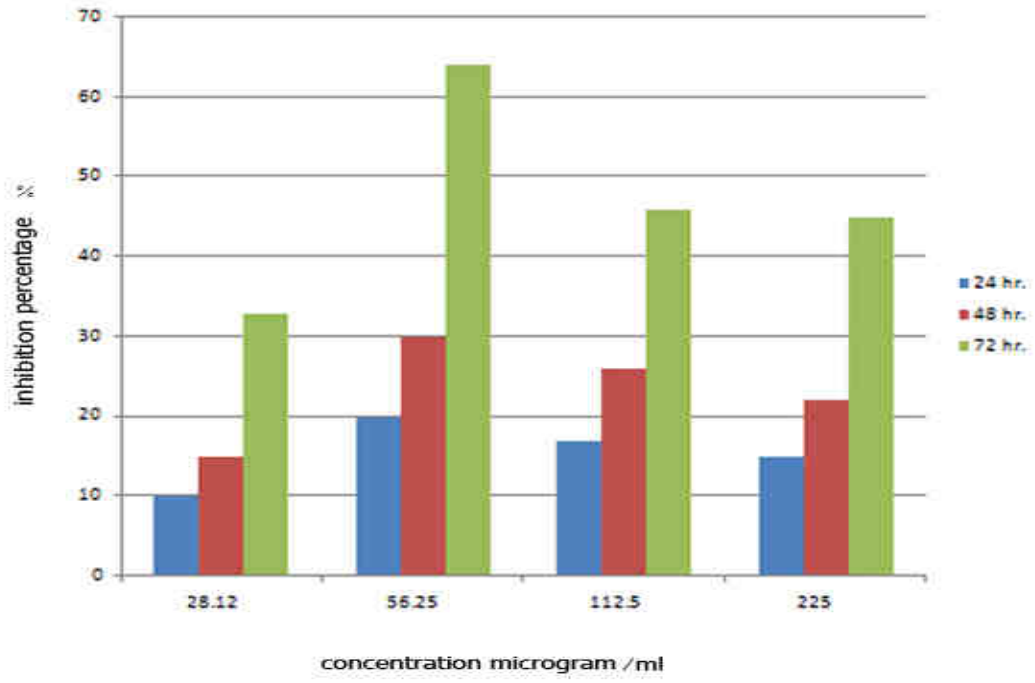

Figure-(3):The cytotoxic effects of purified lectin from *Acinetobacter baumannii* on Hep-2cell line *in vitro* during different exposure times

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