Isolation and Purification of Extracellular Toxic Complex Produced From *KlebsiellaPneumonia* K2and Determined Lethal Dose (LD50) in Mice

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Abstract

Fifty clinical samples collected from sputum of patients pneumonia ,identification by biochemical test and VIETK system were revealed 15 isolate as *Kebsiellaspp,* only 10 isolate represented*K .pneumonia,* The isolate were examined to produce extracellular toxic complex (ETC) it was found that *K .pneumonia* K_2 appear to produce a great deal more toxic ETC had the highest percentage of LPS. Two method of purification this toxin using :First Aqueous two phase systems using Dextran**:** PVP phase(4.25:0.75) the ETC complex came with sodium sulphate solution (0.2M) concentration with protein concentration(97.173 mg/ml **)** in the lower layer and the mice died with 4 hours. The second method by using two step column chromatography, ion exchange DEAE–Cellulose and gel filtration (Sepharose-4B). In the first step sample given lethal activity by injection to the mice after six hour with protein concentration(55mg/ml), More purification by the second step animal died after 3 h with contain protein(27.75mg/ml).TheMolecular weight of ETC was 39810 Dalton determined through Gel-filtration chromatography using Sepharose 6B gel.TheLD50 value of purified toxin was calculated, and the result was (6.52 mg/ml) of toxin .

KEYWORDS*:K.pneumonia* , Purified extracellular toxic complex , Lethal dose 50%, molecular weight

Introduction

Klebsiellapneumonia is responsible for a variety of diseases in humans and animals. It's a prominent nosocomial pathogen mainly responsible for urinary tract, respiratory tract or blood infections (Podschun and Ullmann., 1998). Multiple *Klebsiella*components (e.g., fimbriae , siderophores ,LPS , and capsule) have been considered to be potential virulence factors , Among these factors ,LPS induce TNF-∞ (Henderson., *et al* 1996) . Also , the capsular polysaccharide (CPS) has ability to stimulate antibody production (Fung., *et al* 2000). Infection with *Klebsiella* organisms occurs in the lungs, where they cause destructive changes, inflammation, hemorrhage and necrosis occur within lung tissue, sometimes producing a thick, bloody, mucoid sputum described as currant jelly sputum (Chien-Ko*et al*., 2000) .

The *Klebsiella pneumonia* can cause a lobar pneumonia with extensive pulmonary tissue destruction in normal animals. There is an extracellular toxic substance produced by *K. pneumonia* that is responsible for the lethality and extensive lung pathology. (Domenico*et al*., 1982, Mohammed.,2007)

Lipopolysaccharides (LPS), or endotoxins, are an integral component of the outer membrane of all gram negative bacteria. Approximately 70% of the bacterial surface consists of these molecules, which are responsible for the stabilization and organization of bacterial walls .Bacteria shed lipopolysaccharides into the environment, thus making endotoxins ubiquitous (Boratynski*et al.*, 2004). Lipid A is the most conservative part of LPS and is responsible for the toxicity. Lipid A or endotoxin have particular influence on monocytes and macrophages. Activated cells release mediators, such as tumor necrosis factor (TNF), interleukins (IL), colony stimulating factor (CSF). (Laichalk*et al.*, 1996).

The initial interaction between bacteria and the host mucosal immune system probably occurred through recognition between these surface components and dendritic cells. The mucosal immune response is orchestrated by a network of surveillance based on dendritic cells (DCs), which are professional antigen presenting cells (Cools *et al.*, 2007). The role of DCs in the development of *K .pneumonia* infections has not clearly elucidated. DCs are a critical component of early lung inflammation elicited in a murine *K. pneumonia* infection model and play a role in regulating lung barrier integrity (VonWulffen*et al.*, 2007).

In another murine model of invasive bacterial pneumonia, it has been shown that DCs expressing TLR9 are required for effective innate immune response against *K. pneumonia*, in particular to allow functional activation of lung macrophages and NK and T cells (Bhan*et al.*, 2007). In humans, little is known about the contribution of DCs to the host response to *K. pneumonia* infections. Only *in vitro* experiments have been performed, and they indicate that *K. pneumonia* induces maturation of immature human DCs and production of interleukin 12 (IL-12) (Braat*et al.*, 2004).

The aims of the studyIsolation and purification of extracellular toxic complex (ETC) from local strain of *K. pneumonia*k2 which isolated from sputum of patients complaining from pneumonia by using series method of column chromatography and calculated lethal dose 50% for extracellular toxic complex of *K. pneumonia* .

Materials and Methods

Collection of the samples : During the period of November 2012 –May 2013, 50 samplescollected from sputum of patients which they were suffering from pneumonia. These samples were obtain from Ibn Al Balady hospital and Baghdad hospital . All isolates were identified as *Klebsiella pneumonia*according (Stock and Weidemann, 2001 ., Tortora*et al.*, 2004).

Diagnosis by VITEK 2 System: Followed the steps according to the manufacturer's instructions By Bio-Merieux Company.

Production of the ETC from the *Klebsiella pneumonia* isolate

Fifty ml of sterilized Luria Bertani medium (3.2.4) pH 8.0,was inoculated with isolated colonies of each *K. pneumoniae* bacteria .The bacteria was incubated for 24 hours at 37°C in a shaker adjusted to 200 rpm After incubation, the bacterial growth culture was centrifuged at (10000xg) at 4°C for 20 minutes, then the supernatant fluid was

concentrated against sucrose at 4°C and the final volume reduced to 5 ml by the dialyzing tube.

Assay of lethal toxicity in mice

According to Chhibber*et al*.,(2004) .bacterial toxin 0.5 ml from steps perovisly was injected into ten mice (4 to 6 week-old) interapertonally (I .p) and two mice as control was injected with the same volume of sterilized Tris- HCl buffer only .

Determination of protein concentration

Protein concentration was carried out according to Bradford (1976) by using Coomasie Brilliant Blue G-250 and the absorbance was measured at 595 nm by spectrophotometer.concentration of bovine serum albumin.

Preparation of large (volume) of ETC crude from*Klebsiella pneumonia* K2 isolate

One literof Luria Bertanimedium pH 8.0 sterilized was inoculated with 10 ml of bacterial growth and incubated for 24 hours at 37°C in a shaker adjusted to 200 rpm. After incubation, the bacterial growth culture was centrifuged at (10000Xg) at 4°C for 20 minutes, then the supernatant was concentrated against sucrose at 4°C and the final volume reduced to 50 ml by the dialyzing tube.

Purification of the ETC toxin by Aqueous Two Phase systems

It was prepared according to Dimond and Hus (1989) by using Dextran T-150 system prepared 20% concentration with Polyvinyl pyrrolidone (MW : 25,000-30,000) to final rate 1:1 (wt:wt). Two molar of sodium sulfate were add to the system to get gradient concentrations of salt in range of (0-0.20) M, 0.5 ml of the ETC crud were add to the system. The system that gave best separation were selected. The activity and protein concentration determined in two phases (the upper phase and the lower phase) and the assay of lethal toxicity in mice activity was determined in both phases system.

Ion exchange chromatography with DEAE- Cellulose

it was carried out according to Schutte*et al*., (1997**)**with modification **.**A DEAE-Cellulose, A column (3.5x15cm) was washed equilibration buffer 0.01M of Tris –HCl buffer pH8.0 . Ten millitersof previous dialyzed crude sample was loaded into the column carefully,the flow of elution was adjusted to 30 ml / hr and fractions of 5 ml were collected by means of fraction collector and absorbance was monitored at 280 nm by spectrophotometer. The bound protein was eluted with sodium chloride linear gradient from 0 to 1 M with 0.01Mof Tris-HCl buffer.

Gel filtration chromatography with Sepharose – 4B column

Sepharose - 4B column chromatographyit was prepared according to the instructions of the manufacturing company. The column was equilibrated over night with 0.01M Tris-HCl buffer pH 8.0. Fivemilliliter of the concentrated toxin that been eluted from previously step were applied on the top of the Sepharose -4B column. Fractions which suspected to represent the targeted ETC (after injected into mice to which gave lethal

toxicity) where collected and concentrated with sucrose to final volume of 5 ml to start the second gel filtration analysis. In molecular weight determination, gel filtration for a mixture of standard proteins (Catalase, 232000 Daltons;Bovine serum albumin, 67000 Daltons; Casein, 236000 daltons, Inuline 5000 daltons) was carried out this column in same conditions. The elution volume (Ve) for each one of them was calculated. A standard curve represents the linear relationship between the relative elution volume of each protein and column void volume (Ve/Vo) and the logarithms of their molecular weight were drawn.

Results and Discussion

Isolation of *Klebsiella pneumonia*

The results showed that 15 bacterial isolate from the total 50 sample bacterial isolates were given characterization of *Klebsiellaspp* .which growth on MacConkey agar , ferment lactose sugar and appearance pink shiny Colonies texture were sticky and have a moist mucoid appearance on nutrient agar plates. The *Klebsiella* characterized by inability to analysis the blood ,this characterization used for distinguished *Klebsiella* from other bacteria that similar with bacteria growth at the MacConkey agar but they analyze the blood as a spp. Serratia (Don *et al*., 2005). Also there was a very distinctive pink color due to lactose fermentation on MacConkey agar plates (Flournoy*et al.*, 1990). MacConkey agar is a differential plating medium recommended for use in the isolation and differentiation of lactose-fermenting organisms from nonfermenting gram-negative enteric bacteria. It is selective by the presence of specific inhibitors. The differential action of MacConkey agar is based on fermentation of lactose .Colonies of organisms capable of fermenting lactose produce a localized pH drop which, followed by absorption of the neutral red, imparts a red color to the colony. Colonies of organisms which do not ferment lactose remain colorless and translucent (Flournoy*et al*., 1990). The bacteria were arranged singly, and in pairs . The capsule was clear with capsular stain (Nigrosin stain) .

Klebsiellapneumoniae was diagnosed for other species , according to the results of the VITEK2 device .Ten isolation of bacteria was characterized ***Klebsiella pneumonia*** While were 5 isolates were belonging to ***K.ozaenae.*** it was shown that *klebsiella pneumonia* was the most isolated species that causes pneumonia according to its pathogenic factors that they could possess (Umeh*et al* .,2006).This result is correspond to with another study worked seven Iraqi hospital The percentage of *Klebsiella pneumonia* from clinical isolated about(81.24%)(Najmadeen ,2006)

Production of the Extracellular toxic complex(ETC) from *Klebsiella pneumonia (K2)*

The results of the ETC production revealed that all of isolate *K. pneumonia* from the collected samples . Table (1) shown that the ETC toxin of the isolate K2 were killed the mice when injected within 4 hours , while the ETC toxin which produce from other isolates gave died time during a longer period (5-12) hours. These results means that the ETC of *K. pneumonia* K2 appear to produce a great deal more ETC than other of *K. pneumoniae* isolates and The compositions of the ETCs various indifferent isolate and the most toxic ETC had the highest percentage of LPS and The least toxic ETC had the

lowest percentage of LPS Indeed, there is clear correlation between the toxicity of the ETC preparation and its percentage of LPS (Straus., 1987).

Table (1): The production of ETC toxin produced by *K. pneumonia* isolates

No K. pneumonia	Dose of injection	Time Dead (hours)
***K. pneumonia* k1**	0.5 ml	7 hour
***K .pneumonia* k2**	0.5 ml	4 hour
***K. pneumonia* k3**	0.5 ml	8 hour
***K .pneumonia* k4**	0.5 ml	5 hour
***K .pneumonia* k5**	0.5 ml	7 hour
***K .pneumonia* k6**	0.5 ml	12 hour
***K .pneumonia* k7**	0.5 ml	8 hour
***K .pneumonia* k8**	0.5 ml	12 hour
***K .pneumonia* k9**	0.5 ml	7 hour
***K .pneumonia* k10**	0.5 ml	12hour

Purification of the ETC toxin by Aqueous Two Phase systems

Result showed in the table (2) The two phase system gave higher killing with (4.25:0.75) which contained ETC in the lower layer and the mice died with 4 hours, but the other phase don't gave any activity in both upper and lower phases that means the ETC complex lost its activities when used the higher concentration for the Dextran**:** pvp phase, the ETC complex came with sodium sulphate solution (0.2M) concentration.

Aqueous two phase system (ATPS) is an alternative method for separation of biomolecules which reduces number of steps and thus reduces the overall cost(Naganagouda and Mulimani, 2008) It has many advantages like simple and benign technique (presence of more than 80% water in both phases), rapid separation with little denaturation (volatile organic components are not used), rapid mass transfer (low interfacial tension), selective separation (affinity partition) and easy scale up. (Raja *et al.,* 2011).

Table (2) survival time of ETC in two aqueous phase system Dextran (150000-200000 KD) and poly vinyl pyridine (pvp) .

dextran/pvp: Sodiumsulphate	Protein conc.(mg/ml)		Survival time(hours)	
	Upper	Lower	Upper	Lower
5 : 0	103.04	_	—	_
4 : 1	64.13	93.913	_	12 hr
4.875:0.125	41.086	96.086	_	-
4.750:0.25	99.565	38.043	_	—
4.5:0.5	28.043	47.826	_	_
4.25:0.75	44.78	97.173	_	4 hr
4.125:0.875	67.173	38.695	_	10 hr

Purification of the (ETC)of *K .pneumoniae* K2 by column chromatography:-

Purification of extract of *K .pneumonia* K2 by DEAE- Cellulose

Washing DEAE-Cellulose column by Tris- base and injection in to mice no effect was shown. Elution with liner gradient NaCl (0-1 M) were shown two peak , The first peak was located between (38-41) tubes, those tubes were collected together in one volume, then concentrated against sucrose at 4°C, the final volume reduced to 10 ml. The second peak was located between (44-47) tubes, those tubes were also collected, and concentrated against sucrose at 4°C then the volume was finally reduced to 10 ml. The two peaks were plotted according to their absorbance which were monitored at 280 nm with the spectrophotometer. Figure (1). After the obtaining of two peaks which were formed, survival time of mice injected with the purified material of *K .pneumoniae* K2 were shown in (Table 3).

Table (3) showed the activity of peak –II , had much more lethality on mice than peak -I, so the mice died during sex hours, while mice injected with the sample of the peak1 at 8 hours survived longer. This means that the concentration of extracellular toxic complex (ETC) was in large quantity in the peak –II , while the concentration of the complex compound in peak-I, was low in concentration. In addition, the peak-II contain large quantity of capsular polysaccharide and lipopolysaccharides and protein , so this result indicated a correlation between the concentration of ETC and the survival time of mice(Straus., 1987).This result was in agreement with (Dominco*et al*., 1982.,Straus *et*

al., 1985) . The ETC produced by *Klebsiellapneumoniae* K2 (chosen isolate) was composed of (LPS,CPS, and protein) that were not separable by ultrafiltration or ion – exchange chromatography. Therefore it appears that the ETC is excreted as a complex by this isolate (*K .pneumoniae* K2) in the stationary phase. For this reason the lethality of peak-II showed more effect than the other peak, according to the survival time of animals after injection with the same volume for each peaks. The animals which were injected with extract of peak-I died within 12 hours, but with peak –II takes less time.

Mohammed.,(2007) referred when purified ETC on DEAE-Cellulose Three peaks were formed, each peak was checked for its lethality .The final results showed that animal injected with peak –I sample died after 6 h of injection, and with peak –II animal died after 20 h, while with peak –III the animal died after 39 hours.

Table (3) : The survival time of mice injected with purified toxin of *K .pneumonia* K2

Peak Number	Number of Mice injected (i.p.)	Protein Concentration	Survival time of Mice
Peak No.I	3	22mg/ml	12hr.
Control	3	-	Survived
Peak No. II	3	55gm/ml	6 hr.
Control	3	-	Survived

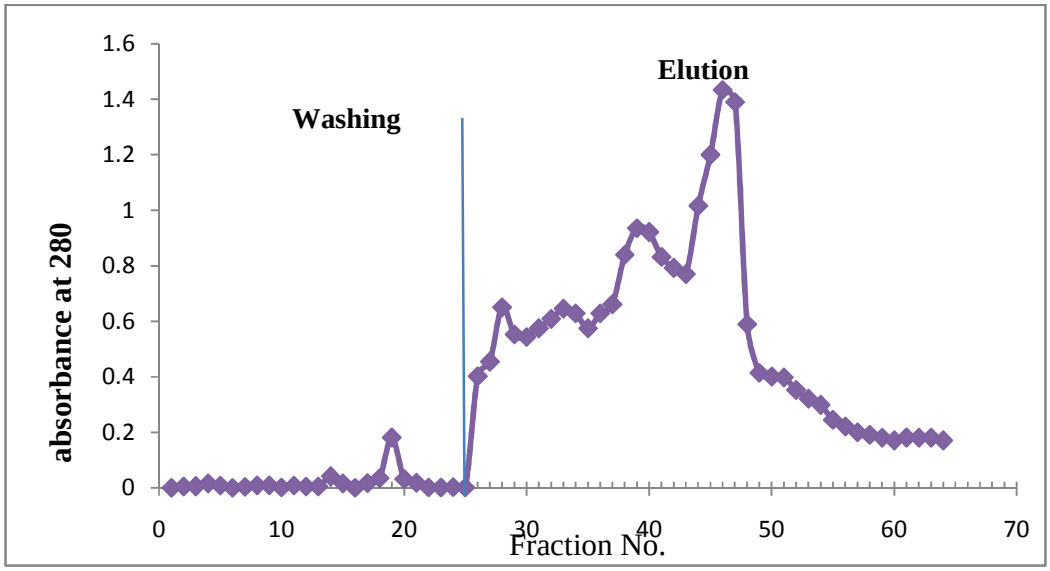

Figure (1) : Purification of extract of *K. pneumoniae* K2 by DEAE- Cellulose column (3.5x12 cm), eluted with Tris-base buffer 0.01M, pH 8.0 at a flow rate of 60 ml /hr.

The collected active fraction from ion-exchange chromatography (15ml) were concentrated against sucrose in order to reduce the volume of the sample to 5ml for gel filtration .gel filtration was used for complete purification of partially purified ETC by applying it to sepharose 4B column because of its fine porous beads. For this reason the Sepharose - 4B gel was chosen in this study.. Figure(4.2)shown one peak located between tube (10-13) those tubes were collected together in one volume, then concentrated against sucrose at 4°C, the final volume reduced to 10 ml. The second peak was located between (21-25) tubes, Those tubes were also collected, and concentrated against sucrose at 4°C then the volume was finally reduced to 10 ml. The two peaks were plotted according to their absorbance which were monitored at 280 nm with the spectrophotometer. The lethality of purified toxin was investigated according to the survival time injected with the purified toxin which was purified with gel-filtration column. In this method of purification.

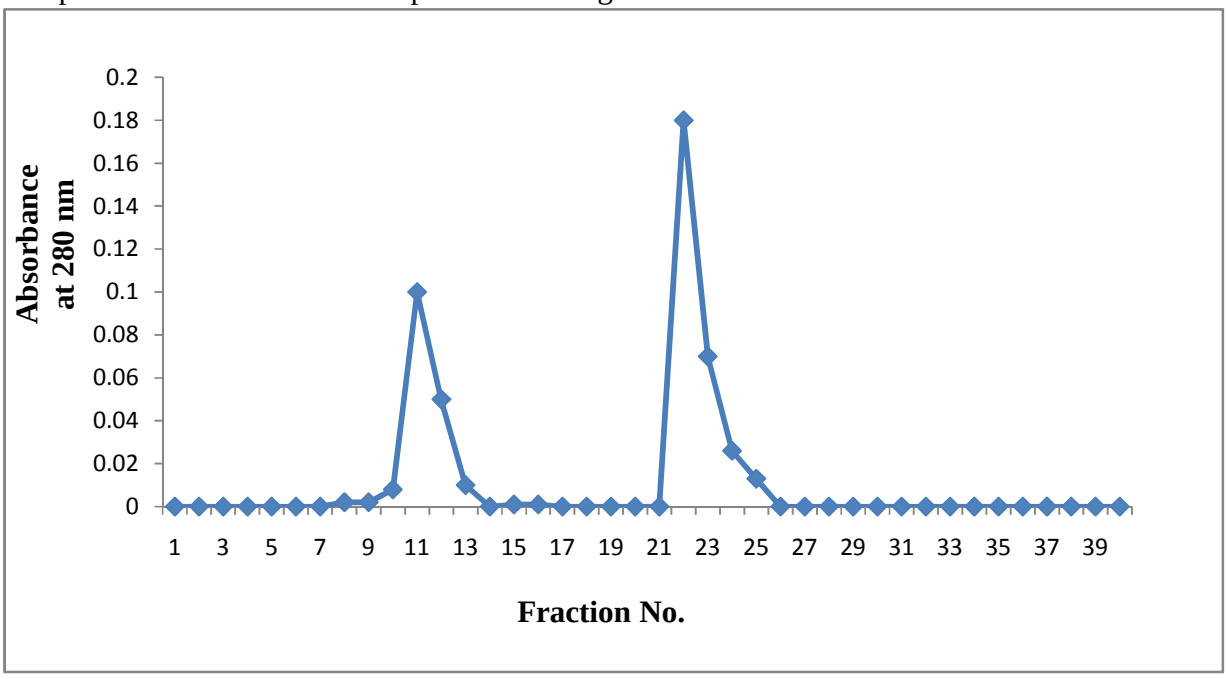

Figure (2): Purification of *K. pneumonia* K2 toxin with Sepharose -4B , Toxin passed through (1.5 x 70 cm), eluted with 0.01M Tris- HCl , pH 8.0 at a flow rate of 60 ml.

Results shown in (Table 4) no effect appeared in peak1,all the mice survived, whereas peak 11 was toxic , all mice dead after three hours.Strau*set al* (1985) referred that ETC could be fractionated by Sepharose- 4B into two distinct pools when the two pools was injected i.p into mice only one was toxic. As well as Al-Jumaily.,*et al* (2012) found that When the extracellular toxic complex (ETC) was purified further by Sepharose -4B column, that means that extract became more virulent on animals. Thus the survival time was less than obtained from DEAE-column.

Table 4: The survival time of mice injected with (ETC) Purified by gel-filtration

sample	Number of mice injected (i.p.)	Protein concentration	Survival time of Mice
0.5 ml purified toxin of peak-I	3	16mg/ml	Survived
0.5 ml control	3	-	Survived
0.5 ml purified toxin of peak-II	3	27.75mg/ml	3hr
0.5 ml control	3	-	Survived

Determination of molecular weight

Molecular weight of purified ETC was the determined using (Sepharose-4B) column as described previously void volume (V_o) of the Column was calculated by estimating the void volume of blue dextran 2000 and the elution volume (Ve) for each standard protein and for the separated fractions of purified ETC.

The ratio of the elution volume of each stander protein and the sample to that of void volume of the blue dextran 2000 was calculated that the (Ve/V_O) of ETC (2.599) and this ratio which located between Bovine serum albumin and casein, hence the molecular weight of ETC was estimated as (39810) Dalton (Figure 2) . Felix *et al* (1986) found the molecular weight of extracellular toxin produced by burn isolate of *klebsiella pneumonia* was approximately 26000 dalton.

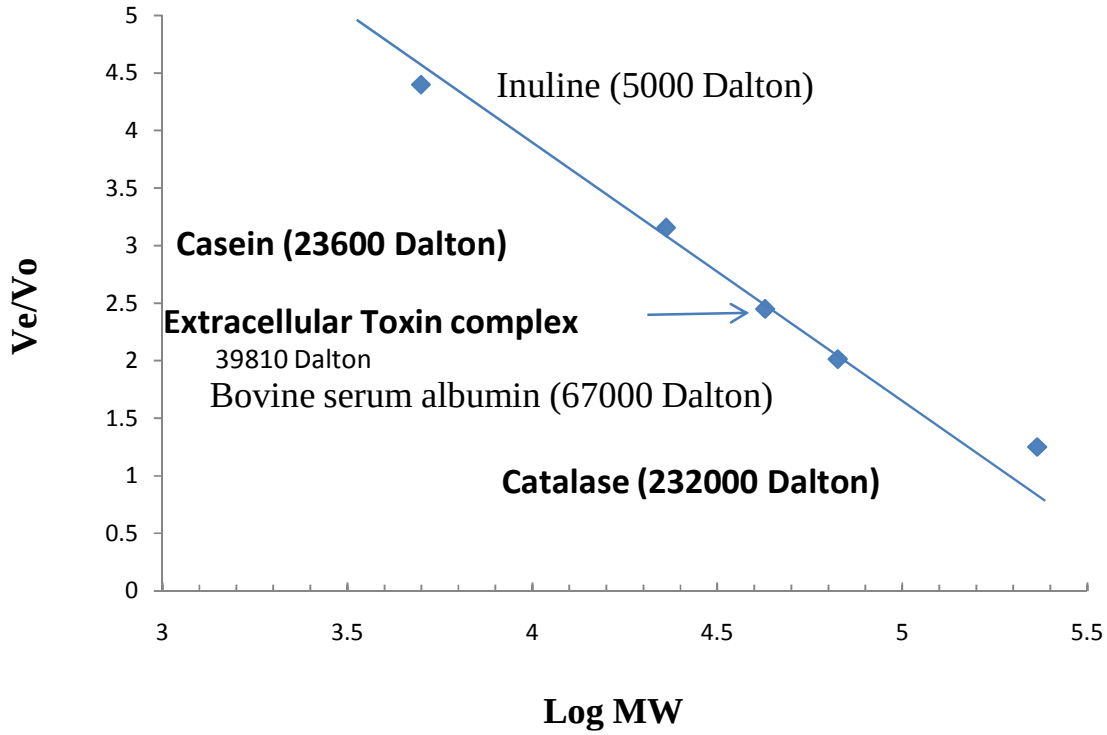

Figure 3 : Linear correlation between the Ve/Vo ratio and Log MW of standard protein molecular weight marker to determine the molecular weight of Extracellular Toxin Complex by using Sepharose - 4B Gel – filtration Column.

LD50% value of purified (ETC) of *K. pneumoniae* K2

Table (5) shown the lethal dose (LD50%) of *K. pneumonia* K2 toxin on mice. From the result had been investigated the concentration (6.52 mg protein/ml) was responsible for the lethal dose of *K. pneumonia K2* to be killed 50% of the total number of mice (8 mice), but when used (10.87mg/ml) pure toxin concentration all the mice were dead (100% of the total number of mice). (Figure4) also for the results could investigated that the(2.17mg/ml) pure toxin gave zero dead mice .

However studies reviewed by Straus *et al*.,(1985) show that injection mice with 1ml of purified toxin (ETC) to gave lethal dose(LD50%). Another study by Mohammed (2007) found that LD50% value of purified to toxin was 0.3 ml (30 mg/ 100ml) of toxin. This quantity was found of effective to cause killing of 50% of the total toxin treated animals.

Table (5): The LD50% value of purified (ETC) of *K. pneumoniae* K2

Protein Con. mg/ml	Number of mice			Percentage of deaths (%)
	Dead	**Lived**	**Total**	
10.87	8	0	8	100.00
8.7	7	1	8	87.50
6.52	4	4	8	50.00
4.35	1	7	8	12.50
2.17	0	8	8	0.00

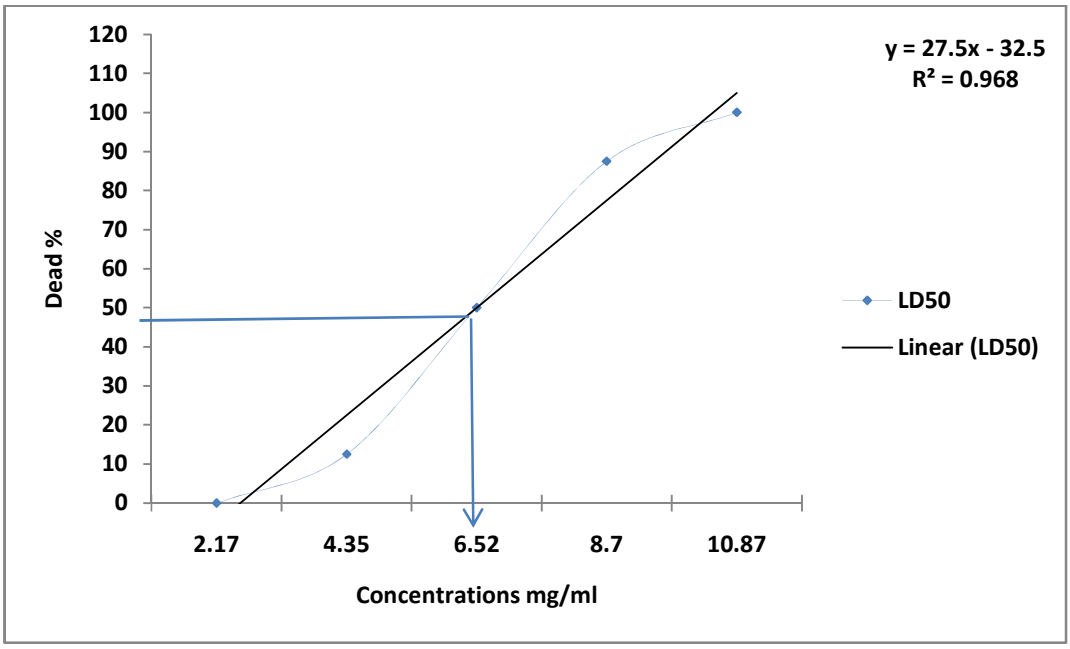

Figure (4): The lethal effect of the concentrations of *K. pneumoniae* toxin on mice.

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