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Purification of hemolysin from *Aspergillus fumigatus* and study its cytotoxic effect on normal cell line (REF) *in vitro*

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ABSTRACT

Aspergillus fumigtus produced a protenaceous hemolysin, when incubated on sheeps blood agar, from 41 isolates thirty isolates (73.1%) has able to produce hemolysin. Hemolysin purified from tryptic soy broth using ion exchang and gel filtaration and found to have amolecular weight of approximately 74.52 KD a. In this study, hemolysin was used in an experimental model to study its Cytotoxic activity by evaluating effect on REF cell line (Rat embryonic fibroblast), on exposure time of 24 hrs at three different concentrations triplicate of each concentration were used, Cytotoxicity of the purified compounds are active against REF cell line under study and a toxic effect was clear with a significant difference at the level of probability ($p< 0.05$) and this effect was increase gradually with the increase of hemolysin concentration.

Keywords: *Aspergillus fumigatus*, Hemolysin,Cytotoxic activity.

INTRODUCTION

Aspergillus fumigatus, apathogenic mould causing a wide range of diseases including aspergillosis, produces a series of toxic substances which appear to act inan additive and/or synergic way on cells.

Hemolysin (Asp-HS). The protein is secreted into the environment and can kill cells that are in the vicinity of the spore. In IA patients this secrete can be found in the urine (Davies, 1991; Arruda *et al.*, 1992a; Arruda *et al.*, 1992b; Kurup *et al.*, 1994; Lamy and; Latgé, 1999). The hemolysin, which enables the fungus to disrupt blood cells, contains negatively charged domains and can also be detected in infected patients. However, despite of the facts that the hemolysin has toxic effects it seems not to be a main virulence factor but a compound that increases the effects of other toxic factors involved in pathogenicity (Ebina *et al.*, 1983; Yokota *et al.*, 1985; Fukuchi *et al.*, 1996; Malicev *et al.*, 2007). The hemolysin produced by *A. fumigatus* (asp-hemolysin) promotes Aspergillosis (Ebina *et al.*, 1982), and may also promote opportunistic infections, (Young *et al.*, 1970).

MATERIALS AND METHODS

Screening the ability of 41 *A. fumigatus* isolates for hemolysin production

Forty one of *A. fumigatus* isolates isolated from patients with aspergillosis from Chect and respiratory diseases specialized center, Ministry of health, Baghdad governorate these isolates grown on potato dextrose agar (PDA), One drop of spore suspension $5*10^6$ *A. fumigatus* of each isolates was inoculated on sheep blood agar (SBA). The plates were incubated at 37 °C for 7 days, triplicate of each isolates. The evidence of hemolysin in the medium indicated by presence of clear hemolysis in the medium and considered as a positive result. Mean of hemolytic diameter were

calculated in each plate (Donohue *et al.*, 2006).

Purification of Hemolysin

Aspergillus fumigatus isolates give high hemolysis on SBA was grown on PDA and the conidia recovered, Approximately $1*10^5$ spores were added to 500 ml of tryptic soy broth (TSB). The cultures were incubated at 23 ◦C for 48 hr on an incubator shaker at 100 rpm. Subsequently, the temperature was raised to 35°C and the incubation continued for an additional 72 hrs. The fungal mass was then removed by filtering through Whatman 541 filter paper in a Buchner funnel. The concentrate from the filtration was then subjected to ion exchange chromatography (Berne *et al*., 2005).

Ion exchange and Gel filtration chromatography

DEAE-Cellulose column (2×23cm) was prepared according to Whitaker and Bernard, (1972) and equilibrated with 20 mM Tris HCl (2.7) buffer (0.05M, pH8.0).

Fraction was plated on SBA and hemolysis noted at 24 hrs. Then these fractions pooled together for the last step of purification by gel filtration chromatography using sephadex G 50.5ml of the protein solution obtained from the elution fractions of ion exchange chromatograph was added gently to column surface and eluted using 0.1 M potassium phosphate buffer pH 8.0 with flow rate of 20 ml/hour (5ml for each fraction). Optical density (at 280nm), Fractions represents protein concentration were pooled and kept at 4°C for further experiments.

Determination the molecular weight of hemolysin using sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis(SDS- PAGE) The molecular weight of protease and hemolysin determined by analyzed the pictures of column in photocapt analysis software (Hassan, 2009).

Subculture of REF cell line:

Single cell suspension was prepared by treating 25 cm^3 tissue culture flask with 2 ml trypsin solution incubated for 2 min at 37°C in an incubator supplemented with (5%) CO_2 after detachment of the cells from the flask surface by gently tapping of the flask followed by the addition of 20 ml of growth medium supplemented with 10% fetal bovine serum, then the viability test of the cells was made by using trypan blue dye which stains the dead cells. Cells suspension was well mixed followed by transferring 200 µl/well to the 96 well flat bottom micro titer plate using automatic micropipette containing ($1x10^5$ cell/well). Plates were incubated at 37°C in an incubator supplemented with (5%) CO_2 until 60-70% confluence of the internal surface area of the well for REF cell line (Freshney, 2000).

Cytotoxicity assay:

To detect the growth inhibition of REF cell line, culture of this cell line was incubated with different concentrations of each compound used in our study these compounds done by different purification technique depend on the nature of the compound, cells were treated and incubated with the purified extract of hemolysin, three concentrations at triplicate form of each extract to investigate the cytotoxic effect of this extract respectively, the concentrations used as follows: hemolysin (2.5, 5, 10µg/ml), triplicate form of each concentration were used. Negative Control was achieved by incubating REF cell line with only maintenance medium.

Detection of the Cytotoxic Effect:

Neutral red assay used to detect the cytotoxic effect. After elapsing the incubation period, 100 µl/well of neutral red dye freshly prepared were added to each well then plates incubated for 2 hrs, viable cells will uptake the dye and the dead not, the plates washed by PBS to remove the excess dye, then 100µl/well

of eluent solution were added to each well to withdraw the dye from the viable cells. Optical density of each well was measured by using ELISA reader at 492nm wave length (Freshney, 2010; Mahony *et al*.,1989).

Percentage of the inhibitory rate was measured according to (Wang *et al*., 2003) as follows:

$$I.R.= \frac{O.D.\ of\ control - O.D.\ of\ test}{O.D.\ of\ control} *100$$

Statistical Analysis:

The values of the investigated parameters were given in terms of mean ± standard error, and differences between means were assessed by analysis of variance (ANOVA) and Duncan test, using SPSS computer program version 7.5. Differences in results were considered significant at probability value equal or less than 0.05 and 0.001(SAS, 2004).

RESULTS AND DISCUSSION

Screening of *A. fumigatus* isolates for hemolysin production.

In order to select the efficient isolate for hemolysin production, screening of 41 *A. fumigatus* isolates screening was done using SBA .Thirteen isolates (73.1%) from 41 *A. fumigatus* has able to produce hemolysin on SBA medium as shown in fig. (1-A), and (26.9%) from 41 *A. fumigatus* isolates which have been unable to produce hemolysin on SBA as shown in fig. (1-B). All the hemolysin production isolates of *A. fumigatus* included in this study give the clear zone but at different ratio.

Table (1) summarizes all information's obtained from calculate the hemolysis diameter of each isolate. Highly significant differences were shown ($P < 0.001$) between the hemolysin producers isolates. Production of hemolysin on SBA medium ranged between (1– 8 mlm).

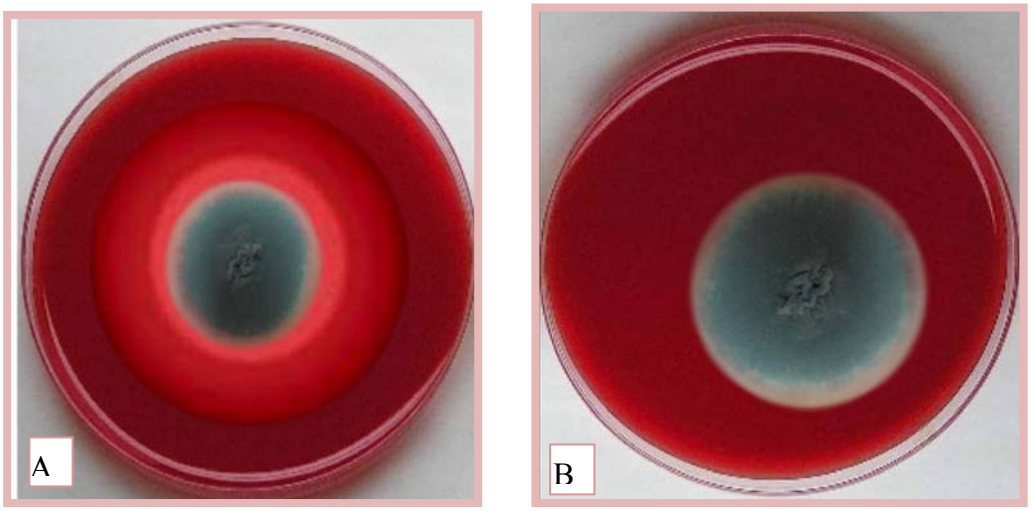

Fig. 1: Growth of *Aspergillus fumigatus* on sheep blood agar (SBA) 7 day, 37°C. A: *Aspergillus fumigatus* produce hemolysin B: *Aspergillus fumigatus* non produce hemolysin .

Isolate AFUI regarded the efficient isolate in hemolysin production and purification by ion exchange and gel filtration chromatography to study affect of hemolysin *in vitro* and selected AFUI for pathological studies since it give highest production.

Hemolysin produced from the strain of *A. fumigatus* isolated from different sources pathogenic and environmental (Ebina *et al*., 1982).

Table 1: Ability of *Aspergillus fumigatus* isolates in production hemolysinon sheep blood agar at 37 C for 7 days.

No. of isolate	Symbol of isolate	Hemolysin prodution±SE mlm
1	AFU1	**8.0 ± 0.5**
2	AFU2	**3 0 ± 0**
3	AFU3	**0 ± 0**
4	AFU4	**4.0 ± 1.1**
5	AFU5	**6.0 ± 0**
6	AFU6	**2.0 ± 0.5**
7	AFU7	**2.0 ± 0.5**
8	AFU8	**4.0 ± 1.7**
9	AFU9	**3.6 ± 1.2**
10	AFU10	**0 ± 0**
11	AFU11	**0 ± 0**
12	AFU12	**3.0 ± 1.0**
13	AFU13	**2.0 ± 0**
14	AFU14	**0 ± 0**
15	AFU15	**7.0 ± 0.5**
16	AFU16	**0 ± 0**
17	AFU17	**0 ± 0**
18	AFU18	**2.0 ± 0**
19	AFU19	**2.0 ± 0**
20	AFU20	**0 ± 0**
21	AFU21	**3.0 ± 1.1**
22	AFU22	**0 ± 0**
23	AFU23	**7.0 ± 1.1**
24	AFU24	**0 ± 0**
25	AFU25	**6.0 ± 0**
26	AFU26	**0 ± 0**
27	AFU26	**5.0 ± 1.1**
28	AFU28	**4.0 ± 0**
29	AFU29	**7.0 ± 1.1**
30	AFU30	**3.0 ± 0**
31	AFU31	**2.0 ± 0.5**
32	AFU32	**5.0 ± 1.1**
33	AFU33	**4.0 ± 0.5**
34	AFU34	**4.0 ± 0.5**
35	AFU35	**2.0 ± 0**
36	AFU36	**3.0 ± 1.5**
37	AFU37	**1.0 ± 0**
38	AFU38	**5.0 ± 1.1**
39	AFU39	**0 ± 0**
40	AFU40	**7.0 ± 1.1**
41	AFU41	**0 ± 0**

significantly different. $p<0.0001$

Hemolysin purification

Results of ion exchange chromatography point out that there was no peak of protein in washing step, while four peaks of protein were separated when eluted association proteins, with the appearance of high protein concentration in peaks from (56-81 fraction) as shown in fig. (2) Hemolysin of high protein concentration peaks in elution steps mean that the hemolysin from *A. fumigatus* had isoforms; in the word the protein purified from elution steps had a negative charge adverse to the ion exchanger, fractions which appeared hemolytic activity on SBA were selected for next step of purification gel filtration.

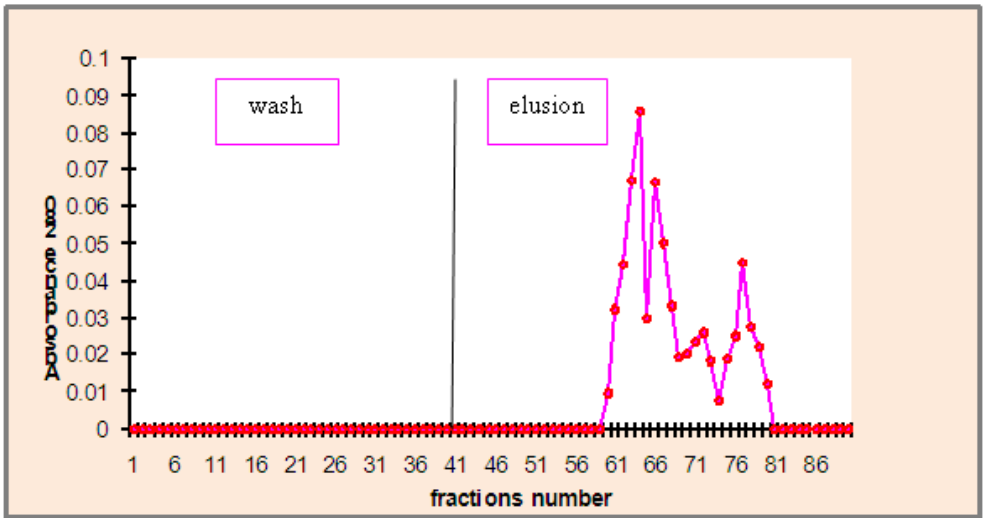

Fig. 2: Ion exchange chromatography for purification of hemolysin produced from *Aspergillus.fumigatus* using DEAE-Cellulose column (2×23 cm) with a flow rate of 20 ml/hour.

Gel filtration chromatography technique was the next step used in the purification of hemolysin produced by *A. fumigatus* after purification by ion exchange chromatography technique. A volume of 5 ml of partially purified hemolysin sparately was applied on Sephadex G-50 column (1.6×24 cm) which previously equilibrated with 0.1 M potassium phosphate buffer (pH 8.0). Sephadex G-50 column which allows ability of separation with high degree of purification (Sivasankar, 2005). Protein was eluted thought the column matrix in a flow rate of 20 ml/hour. Protein peaks were detected by measuring the optical density at 280 nm using UV-VIS spectrophotometer. Results in fig. (3) showed that only one peak (22-31) Fractions included in this peak were pooled, then protein concentration, were measured. Ten microliters of each fraction was plated on SBA and incubated at 37◦C, and hemolysis noted as shown in fig. (4). After purification, hemolysin revealed a clear zone when place on SBA lyses the blood cells underneat Hemolysins lyses RBCs by creating pores or holes in red blood cell membranes resulting in the release of iron that promotes microbial growth (Bullen,1981) Hemolysins are critical virulence factors (Johnson *et al*., 1985; Ou Said *et al*., 1988).

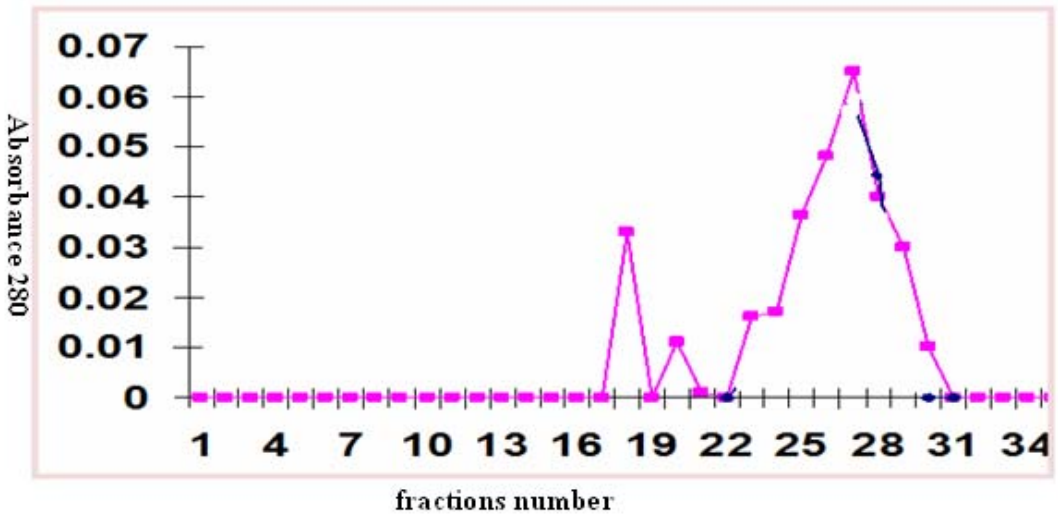

Fig. 3: Gel filtration chromatography for purification of hemolysin extracted from *Aspergillus fumigatus* using Sephadex G-50 column (1.6×24 cm) equilibrated with potassium phosphate buffer pH8.0, fraction volume was 5ml at flow rate of 20ml/hour.

Fig. 4: ability of purified hemolysin fraction of hemolysis in sheep blood agar at 37 ◦C and 24 hrs.

SDS-PAGE analysis

Result in figure (5) and indicates analysis of hemolysin profile of *A. fumigatus* which appeared one band with molecular weight 74.52 KDa agreement with Sonilr (2006) which determines the molecular weight of hemolysin purified from *A. niger* 72 KDa Since hemolysin has an alpha helical structure, it may behave like the alpha helical bundle toxins and like in the molecular weight, e.g., diphtheria toxin (MW 75 kDa), colicins (MW 78 kDa), endotoxins (MW70–135 kDa) and *Pseudomonas aeruginosa* exotoxin (MW 77 kDa) (Parker and Pattus, 1993).

Cytotoxic Effect of Hemolysin on REF Cell Line, *in vitro*

The cytotoxicity effect of purified hemolysin on normal cell line was studied by evaluating its effect on REF cell line (Rat embryonic fibroblast), passage 15 on exposure time of 24 hrs at three different concentrations (2.5, 5, 10 µg/ml) triplicate of each concentration were used by Neutral Red assay.

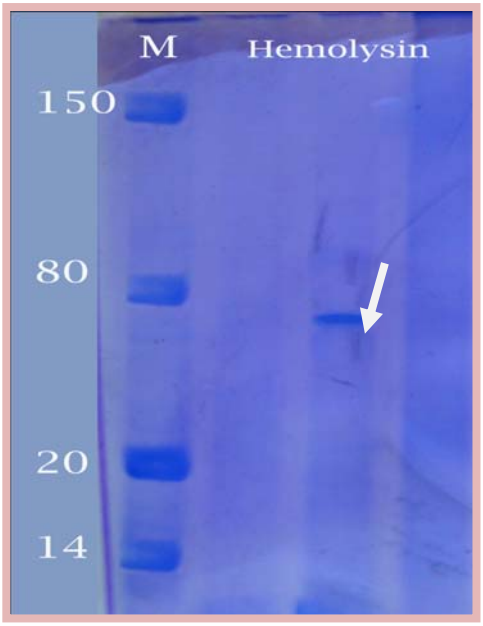

Fig. 5: Protein profile analysis of hemolysin purified from *A. fumigatus* by 10% SDS-PAGE, L1: Protein markers, L2: Pure hemolysin analysis

Cytotoxic Effect of Hemolysin.

Significant cytotoxic effect ($P \leq 0.05$) was observed on growth of REF cell line at the concentrations 2.5, 5, and 10µg/ml with growth inhibition percentage 33.79%, 64.44 % and 76 .68 %, respectively, as shown in the Fig. (6). There was increase in the inhibitory effect when compared with the control (the same cell line without any treatment).

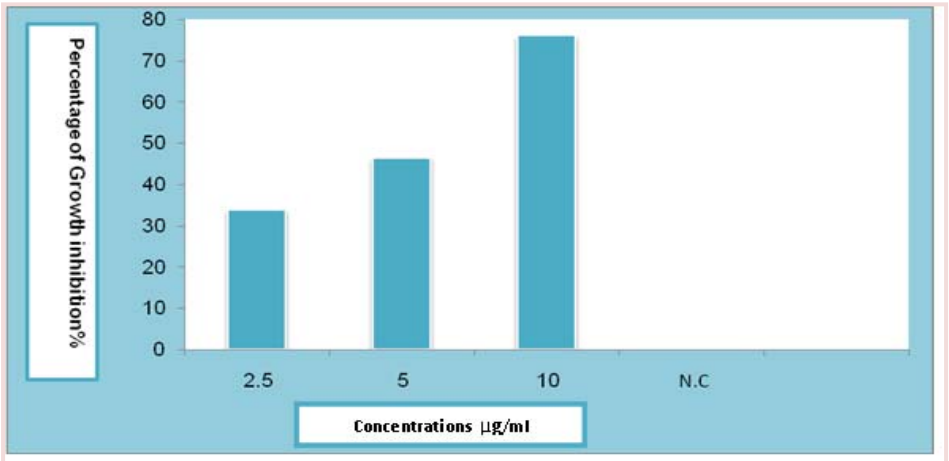

Fig. 6: Cytotoxicity effect of different concentrations of purified Hemolysin from *A. fumigatus* on REF cell line after 24 hr.

Hemolysin is cytolytic and hemolytic protein and can induce effective permeabilization in cell, these activities of hemolysin help us to explain the cytotoxic effect of hemolysin in our result. Hemolysin has an alpha helical structure, Alpha helical toxin cause damage in susceptible cells by creating pores in membranes. (Donohue *et al.*, 2006) experiments showing specific interaction of hemolysin with plasma membrane domains, suggest hemolysin has a specific binding protein, the existence of phospholipid membrane domains involved in cell signaling, endocytosis and attachment of several

toxins and protein indicate that these cells membrane domains probably serve as attachment sites for Hemolysin, leading to their aggregation and formation of the pore (Shepherd *et al*., 1980). Cytotoxicity of hemolysin may be due to the ability of hemolysin to induce DNA damage and mutations (which is considered to be an early event in the process of carcinogenesis) in cell cultures or in animals. It was indicating that the various hemolysin can induce genotoxicity of dietary carcinogens *in vitro* considering that the degree of induction was strongly species dependent (Ebina *et al*., 1982; Ebina *et al*.,1994; Berne *et al*., 2005).

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