

## Study of cytotoxicity of purified toxin A produced from *pseudomonas aeruginosa* on cell lines

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### Abstract:

**P***seudomonas aeruginosa* is the leading etiology of gram negative bacteria causing diseases at most medical centers . The bacterium's virulence depends on number of cell-associated and extracellular factors. Exotoxin A among these virulence factors are toxic for a wide variety of cell line, it inhibits protein synthesis in these lines as well as in numerous organs in animals models. In vitro cytotoxic effects of purified toxin A on two cell lines Rat embryo fibroblast cell line (REF) and on tumor cell line Rhabdomyosarcoma cell line (RD) were studied. Results revealed that toxin A had significant cytotoxic effects on both cell lines.

**Conclusion:** In vitro evaluating the cytotoxic effects of purified toxin A produced from *pseudomonas aeruginosa* at different concentration on normal cell line Rat embryo fibroblast cell line (REF ) and on tumor cell line Rhabdomyosarcoma cell line (RD) revealed that purified exotoxin A has significant inhibitory effects on REF cell line which was dose and time dependent. On the other hand cytotoxic effects of purified toxin A on tumor cell line (RD) reveal that inhibition in cell viability was dependent on concentration of purified toxin A but independent on time.

**Key words :** Toxin A, cytotoxicity assay, cell line

### Introduction:

**E**xotoxin A (ETA) is a heat-labile, single polypeptide chain consist of 613 amino acid and four disulfide linkages with a molecular weight of 66 kilodalton. X-ray crystallography studies and deletion mutation analysis of exotoxin A revealed the overall tertiary structure of the molecule are consists of three functional domains in order to be toxic, namely binding to the receptor on the cell membrane, translocation across the membrane, and ADP ribosylation activities (19).The enzymatic domain transfer the ADP-ribosyl moiety of NAD to a spectrum of cellular targets (3,11). exotoxin A was cytotoxic for variety cell line in vitro by ADP-Ribosulation of elongation factor 2 lead to inhibiting of protein synthesis.

$\text{NAD} + \text{EF-2} = \text{ADP- ribose} - \text{EF-2} + \text{nicotinamide} + \text{H}^+$

Mammalian cells and tissues exhibit a spectrum of susceptibility to *pseudomonas* exotoxin A (5).The initial step in the intoxication process involves exotoxin A binding to specific cell surface receptors followed by receptor-mediated endocytosis. *Pseudomonas* exotoxin A reaches the endoplasmic reticulum following cell-surface binding to a protein receptor a2-macroglobulin receptor (a2-MR)/low-density lipoprotein receptor-related protein LRP1 (4). At a minimum the intoxication process and presentation of the enzymatic activity of *Pseudomonas* exotoxin A in the cytoplasm of target cells can be divided into four main stages: (i) cell surface receptor binding and internalization by endocytosis, (ii) proteolytic hydrolysis by protease furin which cleaves domain II between Arg 279 and Gly 280; (iii) reduction of the Cys265-Cys287 disulfide bond and (iv) transmembrane translocation of the catalytic domain in to the cytoplasmic compartment and finally, enzymatic modification of a cytosolic target (1).

#### Aims of study's

This study was conducted to investigate the cytotoxic and antitumor activities of purified toxin A produced from *Pseudomonas aeruginosa* on different cell lines .

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## Material and methods:

### Toxin A

Toxin A produced from *pseudomonas aeruginosa* strain 26A on dialysate tryptic soy broth and purified by ion exchange chromatography column then by gel filtration sephacryl S 200 was used in this study. Different concentration of purified toxin A (10,20,40,80,100,200) ng/ml that diluted with serum free media were used in this study.

### Viable Cell Count

Viable cell counting were accomplished using trypan blue stain. Cell suspension was prepared at high concentration by trypsinization and resuspension in known volume. The cover slip fixed in its place on a clean haemocytometer. One part of cell suspension 0.2 ml of cells were add to one part of trypan blue 0.2 ml and eight parts 1.6 ml of PBS and mixed together. Transferred 20 µl of the sample to the edge of the cover –slip along running in to counting chamber. After counting by using Light microscope under x40 objective was accomplished. Starting with one chamber of haemocytometer, cells were counted in 1 mm center square, then four 1mm center square were counted and separate count of viable and non viable cells were done. Cell concentration (cell /ml), total cell count and cell viability (%) were calculated.

### Cytotoxicity assay

Cell cultures in microtiteration plate (96wells) were exposed to range of toxin A concentration during the log phase of growth and the effect were determined after recovery time. Cells were collected after adding 2-3 ml of Trypsin –versin not more than few minutes. After trypsinization, cells were counted as mentioned in order to get final concentration 1x10<sup>5</sup> cell /well. Afterward, 200 µl of cell suspension was seeded in each well within sterilized microtiteration plates, the plate was covered with microtiteration lid and sealed with adhesive parafilm then incubated for 24 hours at 37°C. The plates were checked if the cell were formed confluent monolayer and there is no contamination, then the medium was removed and 200 µl of two-fold serial dilutions of purified exotoxin A at concentration 10, 20,40,80,100 and 200 ng /ml were added. Three replicates were used for each concentration. The middle

two columns were used as a control (cells treated with serum free medium only). The plates were incubated at 37°C for the selected exposure time 24, 48 and 72 hours. After exposure, the medium was decanted off and cells in the well were gently washed by adding and removing 0.1 ml of warmed sterile phosphate buffer saline for two times. At the end, 200 µl of crystal violet were added to each well, the plates were incubated at 37°C for 20 min. At the end of incubation, excess dye was removed by washing the well three times with phosphate buffer saline and let it to dry. The absorbance was determined by using an ELISA reader at a wave length 492 nm.

### Cell line

Two cell line are used in this study that include normal cell line (REF) and cancer cell line ( RD) that maintained with RPMI contained 10% fetal calf serum.

## Results:

Studying the inhibitory rate (IR) on Ref cell line at three exposure time 24, 48 and 72 hrs reveal that when the concentration of toxin increase, The inhibitory rate (IR) will increase according to in which at the concentration of 10, 20,40 and 80 ng/ml there is gradual increase in growth inhibition while at the higher concentration of toxin A 100 ng/ml and 200 ng/ml there is significant cytotoxic effect on Ref cell line by 46.76% and 64.35% respectively after 24 hrs of exposure. On the other hand, extended period of treatment to 48 hrs showed that toxin A at concentration 10 ng/ml, 20 ng/ml, 40ng/ml and 80 ng/ml will cause significant increase in IR (p<0.05).The highest inhibition rate 54.8% and 75.80% were obtained by the concentration 100 ng /ml and 200 ng /ml respectively after 48 hrs treatment as seen in figure (1). Treatment for 72 hrs will cause significant increase in IR (p<0.05) in which the highest inhibition (74.12% and 82.55%) were obtained by the concentration 100 ng/ml and 200 ng/ml respectively after 72hrs of exposure as shown in table (1). When the concentration of toxin were fixed at two period 24 and 48 hrs, also 24 and 72 hrs reveal that there is significant differences (p<0.05) during this two period.P3 among all are significant (p<0.05) as seen in table (1).

Table (1) : Inhibition growth rate of REF cell line after exposure to different concentration of purified toxin A at three exposure time (24,48 and 72) hrs.

Conc. of toxin ng/ml	Mean of Percentage of growth Inhibition (IR) within time						P1	P2	P3
	24 hour		48 hour		72 hour				
	Mean (IR)	SE	Mean (IR)	SE	Mean (IR)	SE			
10	14.18	1.16	31.57	5.25	45.10	3.08	0.004	0.003	0.003
20	20.66	1.05	48.41	1.68	57.45	4.02	0.001	0.004	0.001
40	24.48	2.93	56.97	1.21	47.65	2.12	0.001	0.004	0.001
80	30.32	1.41	41.33	1.12	42.35	3.92	0.002	0.005	0.002
100	46.76	0.83	54.08	1.75	74.12	2.12	0.002	0.001	0.001
200	64.35	1.38	75.80	1.87	82.55	1.19	0.002	0.004	0.001

P1: between IR at 24 hrs and IR at 48 hrs, P2 : between IR at 24 hrs and IR at 72 hrs, P3: among all, IR : Inhibitory rate, SE: Standard Error.

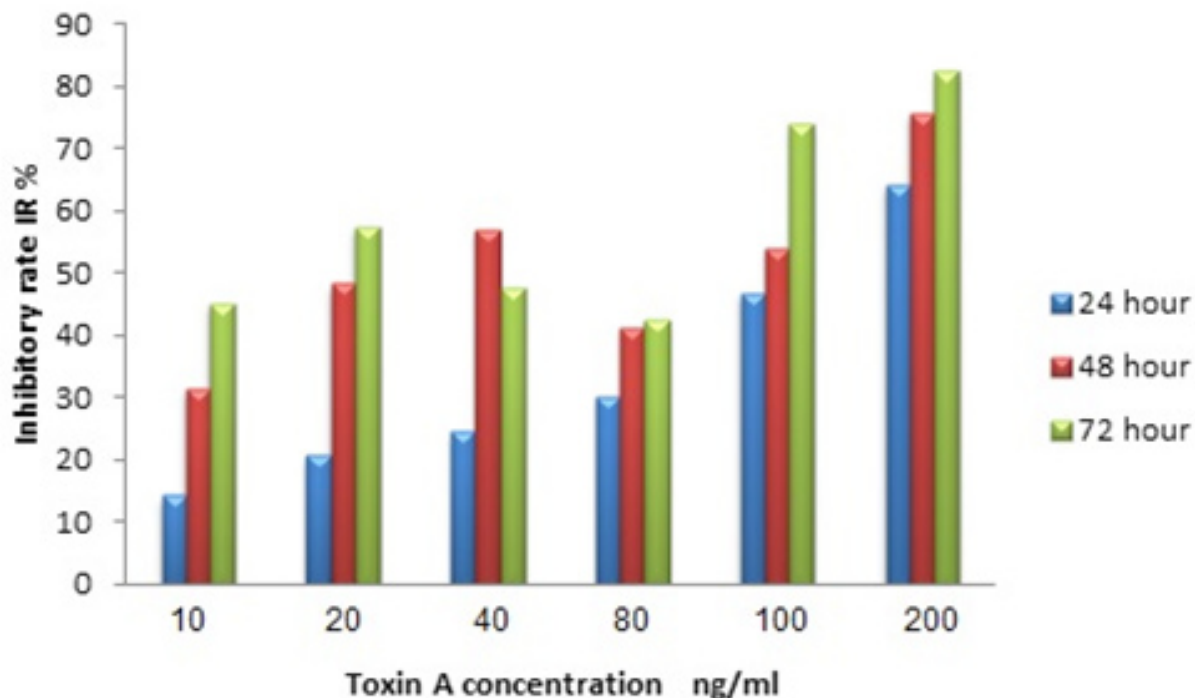


Figure (1) Effect of purified toxin A produced from pseudomonas aeruginosa (26A) strain on the REF cell line

Studying the cytotoxic effect of purified toxin on RD cell line reveal that purified toxin A had a concentration dependent effect on the viability of RD cells. Cell viability decrease significantly with increasing the concentration after 24 hrs treatment as seen in figure (2). The highest significant inhibition in cell viability by 88.40 % and 92.18% respectively were obtained by the concentration 100 ng/ml and 200 ng/ml after 24 hrs. According to the time factor, extended treatment

for 72 hrs showed a time non dependent pattern of inhibition in which the higher inhibitory rate on RD cell line were obtained by the concentration 100 ng/ml and 200 ng/ml after 24 hrs rather than 72 hrs as shown in (figure2). comparison of IR at two period 24 and 48,also 48 and 72 hrs reveal that there is significant differences among all, p3 (p<0.05) as shown in table (2).

Table (2): Inhibition growth rate of of RD cell line after exposure to purified toxin A at different concentration for three exposure time (24,48 and72) hrs

Conc. Of toxin A ng/ml	Mean of Percentage of growth Inhibition (IR) within time						P1	P2	P3
	24 hour		48 hour		72 hour				
	Mean (IR)	SE	Mean (IR)	SE	Mean	SE			
10	32.33	3.05	55.06	0.77	53.09	2.25	0.001	0.001	0.001
20	60.76	0.34	60.35	2.81	70.95	3.41	0.04	0.026	0.044
40	55.67	1.71	59.18	4.99	68.95	3.02	0.007	0.050	0.008
80	64.54	1.71	53.53	4.89	73.46	0.58	0.041	0.003	0.010
100	88.40	1.59	84.12	1.43	73.46	1.53	0.050	0.003	0.001
200	92.18	1.48	76.35	1.24	81.30	3.94	0.004	0.02	0.012

P1: between IR at 24 hrs and IR at 48 hrs, P2 : between IR at 24 hrs and IR at 72 hrs, P3: among all, IR : Inhibitory rate, SE: Standard Error.

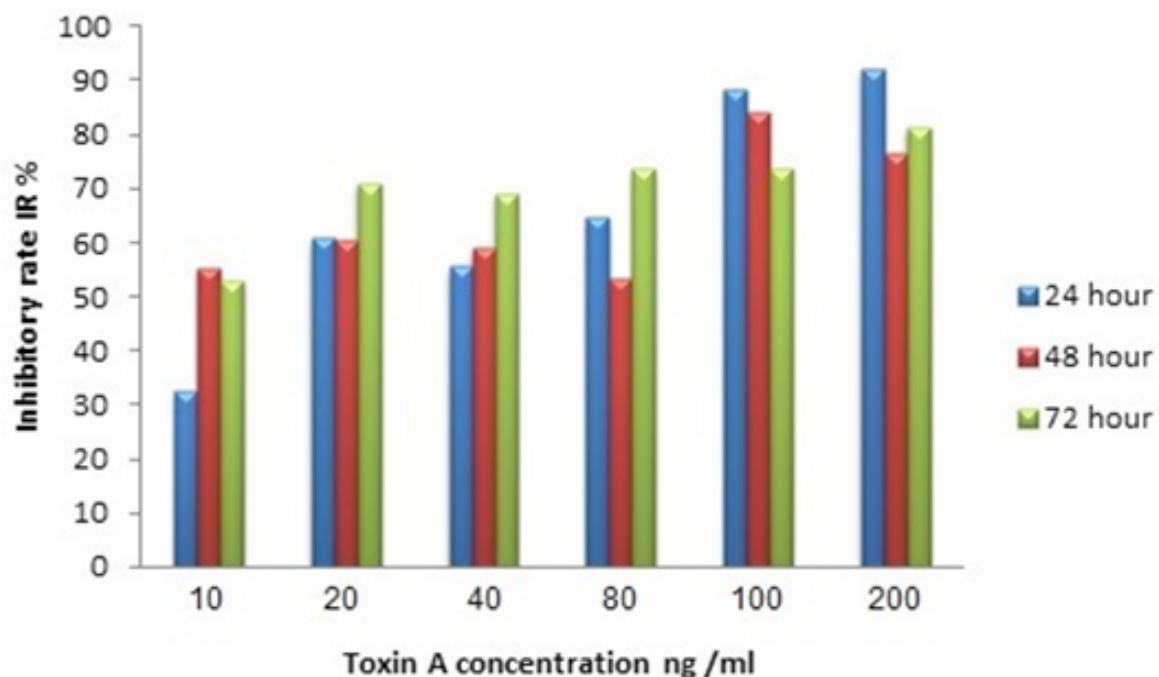


Figure (2) : Effect of purified toxin A produced from *pseudomonas aeruginosa* (26A) strain on viability of RD cell line

## Discussion:

There is strong relation between adherence and cytotoxicity of *P. aeruginosa* isolates. Normal mouse fibroblast and epithelial cell are sensitive to *Pseudomonas* exotoxin A because type III secretion system is contact-dependent in *P. aeruginosa* cytotoxicity and was affected by changes in pH and temperature (8). *P. aeruginosa* cytotoxicity decrease when the pH of medium increased. This may be due to a decrease in adherence of bacteria at high pH levels (18). Previously it was reported that there was an overall decrease in *Pseudomonas* adherence to epithelial cells with increasing pH and that adherence at pH 7 was significantly higher than that measured at all other pH levels and this may explain the reduced cytotoxicity observed as pH increased (6). Type III secretion system (TTSS) is contact dependent system that depend on the number of *P. aeruginosa* that adhering to the eukaryotic cells which are correlated well with the amount of translocated effector proteins toxin A (2). In addition, TTSS protein (toxin A) of *P. aeruginosa* was highly sensitive to environmental conditions, such as temperature, pH, and Ca<sup>2+</sup> level, as well as to metabolic balance Concerning temperature changes (10). The highest cytotoxic effects of purified toxin A were observed at 37°C. This may be explained by finding the genes encoding TTSS are induced at a temperature of 37°C (7). Moreover,

the influence of incubation temperature will relay on effector toxin A protein reveal that maximum effector protein synthesis occurs at 37°C. Exposure of LM fibroblast cells to an acidic extracellular pH did not overcome the protection afforded by ammonium chloride against exotoxin A cytotoxicity. This suggest that sensitive and resistant cells may internalize exotoxin A in a similar manner, the toxin entering the cytosol from a prelysosomal acidic vacuole (14). The Sensitivity of both tumor cell line to purified toxin A is due to Presence of wide array of biologically active surface molecules on cancer cells, and several of these function as receptors for various ligands. They include MHC, or in the case of humans, HLA antigens, cytokine receptors, cell-adhesion molecules, growth factor receptors, Fas-Fas-ligand molecules and others. Their expressions are a subject to alterations, usually to the advantage of tumor growth and spread. Toxin A have several molecular strategies which developed under evolutionary pressure for effective killing(9). Toxin A is cytotoxic for a variety cell line and specifically inhibit protein synthesis in vivo and in vitro by ADP- ribosyl transferase activity on elongation factor 2(13). Multiple assay end point to investigate the effects of incubation time and dose on cytotoxicity of toxin A on tumor cell line and show that cytotoxicity produced by toxin A in many tumor cell line (mylosarcoma, mammary adenocarcinoma, glioma) is dose dependent but its reveal different sensitivity within time(20). The toxic material produced by

*Pseudomonas aeruginosa* designated toxin A produced during the growth of *pseudomonas* strain PA 103 were tested on HEP-2 monolayer cultures and reveal that toxin A was produce significant cytopathic effect in HEP-2 cell line in which the inhibitory rate on tumor cell line may depend on number of tumor cell receptor which internalized the toxin(17). Toxin A from *pseudomonas aeruginosa* will reduces chemoresistance of oral squamous carcinoma cell line via inhibition of

heat shock protein 70 (15). Also toxin A of *pseudomonas aeruginosa* exhibited potent cytotoxicity for many cancer cell lines that specifically target tumor related antigen and was active at concentrations as low as 10 ng/ml (12). Schappa (16) reported that hemangiosarcoma and cancer stem cells sub population are effectively killed by toxin A targeted through epidermal growth factor and urokinase receptors.

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# دراسة التأثير السمي للذيفان A النقي والمنتج من بكتريا *pseudomonas aeruginosa* على خلايا الزرع النسيجي

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## الخلاصة:

تعد بكتريا *pseudomonas aeruginosa* المسبب الرئيسي للإصابات الناتجة عن البكتريا العسوية السالبة لصبغة جرام في معظم المراكز الطبية حيث تعتمد امراضية البكتريا على عدد من عوامل الضراوة المرتبطة بالبكتريا وكذلك المنتجة خارجها. يعد الذيفان A أحد عوامل ضراوة البكتريا والذي له تأثير سام على عدد كبير من خطوط الزرع النسيجي حيث يقوم بتثبيط عمليات انتاج البروتين في تلك الخطوط وفي عدد من الأعضاء في الحيوانات المحقونة تجريبياً. تم دراسة التأثير التثبيطي للمعاملة بالذيفان A على خطين أحدهما خط خلايا الجرذ الجنينية مولدة الألياف REF وخط سرطان الحوض للنساء RD حيث اظهرت النتائج أن الذيفان يمتلك تثبيطاً معنوياً ضد كلا النوعين.