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PRODUCTION AND PURIFICATION OF EXOTOXIN A EXTRACTED FROM SOCIAL STRAINS OF *PSEUDOMONAS AERUGINOSA* IN IRAQ.

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ABSTRACT
The pathogenicity of *Pseudomonas aeruginosa* depended on several virulence factor and among them is a toxic products excreted by *Pseudomonas aeruginosa*, termed exotoxin A, that causes the ADP ribosylation of eucaryotic elongation factor 2 resulting in inhibition of protein synthesis in the affected cell. Exotoxin A from *Pseudomonas aeruginosa* has molecular weight (Mw 65.12KD) has been produced in dialysate tryptic soy broth. Purification of toxin by two steps include ion-exchange column then by gel filtration column to yield highly purified toxin then detection of molecular weight of purified toxin. This study was conducted on extracted and purification of exotoxin A produced by social strains of Pseudomonas *aeruginosa* in Iraq then detection the molecular weight of toxin.

KEY WORDS: Exotoxin A, gel filtration, ion-exchange column, molecular weight, *Pseudomonas aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous, gram negative, water-borne bacterium which may thrive in water and other environments (Reuter *et al*., 2002) it's an opportunistic pathogen that causes serious and sometimes fatal infections in the compromised host, especially in patients with major trauma or thermal injures (Shahanara *et al.*, 2007).

It may form a biofilm of cells on the mucous membranes of the lungs in cystic fibrosis patients and on many other surfaces (Chotirmall *et al.*, 2012; Meluleni *et al*., 1995). Exotoxin A (ETA) is considered as the most toxic virulence factor secreted by *P. aeruginosa*. The toxin was first discovered and purified by Liu who reported that *P. aeruginosa* PA103, a protease-deficient strain, produces more toxin than do strains of *P. aeruginosa* which actively produce proteases (Susan *et al*., 2005; Stephen *et al*., 1988). Exotoxin A appears to mediate both local and systemic disease processes caused by *Pseudomonas aeruginosa*. It has necrotizing activity at the site of bacterial colonization and is thereby thought to contribute to the colonization process. Toxigenic strains cause a more virulent form of pneumonia than non-toxicogenic strains. In terms of its systemic role in virulence, purified Exotoxin A is highly lethal for animals including primates (Boudenet *et. al*., 2004).

ETA is an ADP-ribosylating toxin that catalyzes the transfer of the adenosine diphosphate ribose moiety (ADP-ribose) from NAD to eukaryotic elongation factor 2, resulting (ADP-ribose-EF-2) complex is inactive in the inhibition of protein synthesis and ultimately cell death (Jorgensen *et al*.,2005; Judith *et al* .,1991) . The target residue in eEF2, diphthamide (a modified histidine), spans across a cleft and faces the two phosphates and a ribose of the non-hydrolysable NAD+ analogue, beta TAD. This suggests that the diphthamide is involved in triggering NAD+ cleavage (Melanine *et al*.,2006)

NAD + EF-2 $\rightleftharpoons$ ADP-ribose-EF-2 + nicotinamide + H^+.

ETA is a heat-labile, 613-amino-acid single polypeptide chain with a molecular weight 66-kDa .X-ray crystallography studies and deletion mutation analysis of ETA revealed three structural domains (Guadarrama *et al*., 2005). Domain I of ETA includes aa 1 to 252 (Ia) and 365 to 395 (Ib) and is associated with binding to the receptor of target cells. Domain II, aa 253 to 364, is believed to be involved in translocation of a 37-kDa enzymatically active fragment of ETA across the membrane of the endocytic vesicle to the cytoplasm of the target cell. Domain III, aa 396 to 613, constitutes the enzymatic portion of ETA (Ira, 2003).

Toxin A is produced *in vitro* by most clinical isolates of *P. aeruginosa* and appears to be produced in humans during *P. aeruginosa* infections. In native form, toxin lacks full enzymatic activity. Maximal ADP ribosylation activity is expressed when toxin is denatured and reduced or when it is cleaved by *Pseudomonas* proteases to yield an enzymatically active 37-kDa fragment (Josphen *et* al., 2001). PEA is secreted as, which is extensively modified by target cells in order to generate and deliver the activated 37-kDa enzymatic fragment to the cytosol. The initial step in the intoxification process involves PEA binding to specific cell surface receptors followed by receptor-mediated endocytosis (Chotirmall *et. al*., 2012). A cell surface PEA binding protein was isolated from mouse fibroblasts and liver

cells and subsequently identified as the low-density lipoprotein (LDL) receptor-related protein (LRP) (Judith *et al.*, 1991).

Efficient excretion of toxins plays a key role in the ability of pathogenic microorganisms to cause disease, since these factors can act on targets that are distal from the site of colonization (Susan *et al*., 2005). Extracellular localization of proteins by gram negative bacteria may involve a mechanism which could be fundamentally different from the normal secretion machinery (Aufaugh *et. al*., 2010). Excretion involves transfer of a polypeptide from the site of synthesis across the cytoplasmic as well as the outer membrane, the latter lacking a source of energy and proton motive force. While the outer membrane contains porins which allow free passage of hydrophilic solutes, the size of the pore is too small to allow free diffusion of large proteins from the periplasmic space (Jorgensen *et. al*., 2005). This study was conducted on extracted and purification of exotoxin A produced by social strains of *Pseudomonas aeruginosa* in Iraq then detection the molecular weight of toxin

MATERIALS AND METHODS

Sample Collection

During this survey a total of 123 samples were collected from patients suffering from burns, wounds, otitis media, UTI infection in Al-khademia, al-karej, and Al- Yarmook hospitals in Baghdad, during the period from 1/3/2011 to 30/6/2011.

Samples were taken using asterile cotton swaps while sterile container was used for UTI infection. The Samples were directly streaked on MacConcky agar and incubated at 37°C for 24 hours.

Identification of *pseudomonas aeruginosa*

Morphological Characteristics

Colonies that grow on the selective media MacConkey agar were further identified by studying their morphological characteristics beginning by staining with Gram stain and appearance under light microscope (Gram reaction, shape, arrangements, and spore formation).

Isolation of pseudomonas *aeruginosa*

Non lactose ferment colonies were sub cultured further on cetrimide agar and incubated at 37°C for 24 hours.

Biochemical Tests

The following biochemical tests were achieved for the suspected colonies and as follows: Indole Production Test, Methyl Red Test, Voges-Proskaur Test, Citrate Utilization Test, Urease Test, Triple Sugar Iron Test, Glucose Fermentation Test, Oxidase test, Catalase test, Identification of the isolates further was carried out by sub-culturing representative colonies from MacConkey Agar plates on APi 20E microtubes system.

Production of pyocyanine pigment

For detection the ability of bacteria to produce pyocyanine pigment, selected isolated colonies were streaked of on cetrimide agar then incubate at 37C for 24 hours, the appearance of green colour on plate indicate positive results.

Ability of Bacteria to growth at 42^{o}C

For detection the ability of bacteria to growth at 42^{o}C, it was inoculated on nutrient agar plate and incubated at 42^{o}C for 24 hours, and then appearance of growth at this temperature indicates positive result.

Protease test

Pseudomonas aeruginosa isolate were streaked on skim milk agar to detect their ability to produce protease enzyme.

Production, concentration, precipitation of toxin A from pseudomonas aeruginosa Strains

Production of toxin A.

Media of various composition were used for production of toxin A. a dialyzed medium of Tryptic soy broth was made by suspension of (30) g of the powder in (100)ml of distilled water, then overnight dialysis against 0.01M Tris – OH buffer at 4^{o}C. The dialysate that came out of the dialysis bag was distributed in 1L quantities flask and autoclaved for (15) mint. monosodium glutamate (0.05)M was sterilized by filtration through aseitz filter (0.45)Mm were added aseptically to the dialysis medium with glycerol(1%) after dialysis media came out of the autoclave, then two liter of the medium was distributed in (12) flask .

The organism used as inoculum was previously grown on blood agar plate at 37°C for 18 hours and scraped off with a sterile loop to made cell suspension 10^{10} cell/ml. Culture were started by placing (0.4) ml of the bacterial suspension freshly prepared in each flask, then shaken (120) cycle /min. at 32°C for (18-22) hours. At the end of incubation, cultures were centrifuged at 10,000 xg For 20 min.

Concentration of toxin A

Immediately after centrifugation (1/20) volume of (1) M zinc acetate was added to the supernatant fluid. The mixture was left at 4c for few hours, then centrifuge at 5,000xg for 20 min. to collect the precipitate which was dissolved in 0.3M of sodium citrate solution. The mixture was dialyzed against 0.01M Tris buffer pH 8.0 at 4°C for 24 hours with several changes of the buffer, centrifuge to remove the insoluble material .then supernatant will contained the toxin which referred to as stage (I) toxin.

Preciptation with ammonium sulphate

Toxin was precipitated by addition of $(NH_4)_2SO_4$ at 20%, 40%, 60%, 80% saturation , PH was maintained at 8.0 with concentrated NaOH during the addition. The precipitate was collected by centrifugation, suspend in (20) ml Tris buffer and dialyzed again. The toxin was kept frozen at (-20°C) for (6-8) month without loss of potency.

Purification of toxin by Ion exchange chromatography.

It was carried out according to Whitaker and Bernard, 1972 with modification. A DEAE-cellulose column 1.5 x 35 cm washed with equilibration buffer 0.01 M Tris-OH pH 8, for several times. Ten milliliters of toxin A was applied to the column. The bound toxin was eluted with NaCl linear gradient from 0.1 to 0.5M with buffer 0.01M Tris-OH pH8.0. Toxin was collected at flow rate 3ml /fraction then the absorbance was detected at 280nm using spectrophotometer.

Gel Filtration Chromatography with Sepharose 6B

Sepharose 6B gel column 1.5 x80 was prepared as recommended by Pharmacia Fine Chemicals Company. The column was washed and equilibrated with 0.2M Tris –OH buffer pH 8. Two milliliters of the concentrated toxin purified by DEAE- Cellulose column was applied on to the top of the Sepharose 6B column. Elution was achieved at a flow rate of (3ml/fraction) using the same buffer of equilibration then absorbance of each fraction was measured at 280 nm. The peaks were collected then concentrated by lyophilization then checked for cytotoxicity by injection in mice.

Determination of molecular weight of toxin

Molecular weight of purified toxin was determined by gel filtration chromatography pure toxin A was left to flow through a glass column(1.5x8.0) cm packed with Sepharose 6B,then eluted in a flow rate(3ml /fraction) . Standard protein (Trypsin, bovine serum albumin, ovalbumin, Aldolase) were also applied separately through the column in same conditions. The elution volume (Ve) for each one of them was calculated. A standard curve represents the linear relationship between Ve/Vo against the logarithms of their molecular weight were drawn.

Determination of Protein Concentration

Protein concentration was determined according to Bradford, (1976) and as follows:

A standard curve of bovine serum albumin was carried out by using different concentrations from BSA stock solution according to the volumes. Then (2.5) ml of Coomassie brilliant blue G-250 dye was added, mixed and left to stand for 2 min at room temperature.. The absorbance at 595 nm was measured; the blank was prepared from (0.1) ml of Tris-oH buffer and 2.5 ml of the dye reagent. A standard curve was plotted between the BSA concentrations against the corresponding absorbance of the bovine serum albumin. The protein concentration of toxin sample was estimated by taking (0.1) ml of toxin solution dissolved in Tris-oH buffer, subjected to the same previous addition and read the absorbance at 595nm.

Experimental Animals

Toxicity in mice

Group of Swiss albino BALB/c mice, which were obtained from the ICCMGR, were used in this study for detection the cytotoxicity of exotoxin A. The total (28) mice ages ranged between (8-12) weeks and weighting (25-30) gram were divided in to (4) groups, the last one left as control. Each group was kept in a separate plastic cage, and then the cages were kept in animal house at (23-25) °C temperature. Mice were fed with suitable quantity of water and complete diet. three toxin From three different protease negative strains were used. The animals were treated with (0.5) ml of crude exotoxin A that administrated intra -peritoneally, while the control group administrated (0.5) ml of sterile PBS. The animals were observed for five days, and record the result of death.

RESULTS AND DISCUSSION

1 - Isolation and identification of Pseudomonas *aeruginosa*.

Results have shown that among the total of 161 clinical samples that were collected from different cases of (burns, wounds, otitis media and UTI) infection, only 123 isolate (76.3 %) give typical morphological characteristic and biochemical test that related to *Pseudomonas aeruginosa* , while the rest 38 isolates may belong to other pathogenic bacteria from different genera as shown in table (2).

Table 2. Source of isolation of *Pseudomonas aeruginosa* and their ratio.

Source of sample	No of sample	No of positive strains	Percentage	Protease negative strains	Percentage
Burns	70	63	51.21	1	0.81
Wounds	25	21	17.o8	0	0
Otitis media	36	27	21.95	2	1.62
UTI	30	12	9.75	0	0
Total	161	123	100	3	2.43

Identification of bacterial isolates

Diagnosis of *P. aeruginosa* infection depends upon isolation and laboratory identification of the bacterium. It is identified on the basis of its Gram morphology, and appears as a straight or slightly curved, non-sporulating, motile gram-negative rod that grows aerobically .Morphological characteristics on the suspected isolates were done depending on the colonial shape that it form colorless colonies, 1-3 mm in diameter when cultured on the surface of MaConkey agar. Clinical samples, in general, yield one or another of two smooth colony types. One type has a fried-egg appearance which is large, smooth, with flat edges and an elevated appearance. Another type, frequently obtained from urinary tract infections, has a mucoid appearance. It grows well and commonly is isolated on blood agar and it appears beta hemolytic on blood agar. *P. aeruginosa* colonies.

Biochemical tests

According to the results of morphological and microscopic characteristics, bacterial isolate. Were subject first to number of biochemical tests, that revealed that only 123 out of 161 isolates were related to suspected to belong to *Pseudomonas aeruginosa spp.* they Give positive results for oxidase, catalase, citrate utilization test, but give negative resuls in indol production, methyl red- voges proskour tests, furthermore the isolate give variable results in gelatin liqifaction tests. In Api 20E system, rains give typical results related to *Pseudomonas aeruginosa* .

Ability to grow at 42^{o}C

Colonies streaked on nutrient agar at 42^{o}C for 24 hours, were able to grow at this temperature, the appearance of growth indicate positive results.

Production of protease enzyme

From 123 bacterial isolates that related to *Pseudomonas aeruginosa* spp, 97.57% of isolates were positive for protease ,only 3 isolate 2.47% were negative for protease production as shown in table (2).

Production of toxin A

The medium of choice for the cultivation of *P. aeruginosa* and production of PE for these and other studies as well is the dialyzable portion of Trypticase soy broth (TSBD) ,using proper conditions for growth and medium supplements such as glycerol and monosodium glutamate, substantial quantities of PE can be recovered from the supernatant fluid.

Results show that production of toxin A in tryptic soy broth dialysis with change the concentration of monosodium glutamate from (0.05 to 1) M Will increase OD of toxin, also increase Killing rate when injected to mice. Strains (26A) ,11 that grow on tryptic soy dialysate with the addition of (1)M of monosodium glutamate give higher killing rate than the same strain grow on same media but concentration of monosodium glutamate was(0.05). Strain (24) given the same killing rate when grow in both as shown in table (3,4).

Table 3. Production of exotoxin A in tryptic soy agar dialysate with (0.05)M of monosodium glutamate and precipitation for 2-3 hr.

No. of group	No. of strain	Type of media	Conc of monosodium glutamate(M)	Precipitation with Zinc sulphate (hours)	Injection of other material	Killing time (hr)	Percentage
1	26(1)	T.D.B	0.05	2-3	-	24	4 /5
2	11	T.D.B	0.05	2-3	-	48	/5 3
3	24	T.D.B	0.05	2-3	-	48	/5 2
4	Control		-	-	PBS	120	0/5

Table (4). Production of exotoxin A in tryptic soy agar dialysate with (1)M of monosodium glutamate and precipitation for 24 hr.

NO of group	NO of strain	Type of media	Conc. of monosodium glutamate(M)	Precipitation with Zinc sulphate (hours)	Injection of other material	Killing time	Percentage
1	26(1)	T.D.B	1	24	-	24	5/5
2	11	T.D.B	1	24	-	48	4/5
3	24	T.D.B	1	24	-	48	2/5
4	control		-	-	PBS	120	0/5

Zinc acetate precipitation.

Zinc acetate precipitation of protein extract were used for two time .results show that precipitaton with this salt for 24 hr, will give higher protein concentration and higher toxicity than toxin precipitated for 2-3 with this salts, as shown in table (3,4) .

Ammonium sulphate precipitation

Ammonium sulphate precipitation was achieved using different percentage of saturation ratios ranging between 20% and 80% to concentrate the toxin produced by pseudomonas *aeruginosa.* Results showed that increase the concentration of Ammonium sulphate will increase the precipitation of toxin (OD), also increase the cytotoxicity (killing rate) of toxin extract when injected to mice as mention in table (5).

Table- 5: saturation of toxin with ammonium sulphate.

NO of group	Saturation with ammonium sulphate %	Amount of ammonium sulphate (gm)/100 ml	O.D (0.1)ml	Injection of other material	Killing time(hours)	Percantage
1	0-20	10.6	0.250	-	24	0/5
2	20-40	11.3	0.278	-	24	2/5
3	40-60	12	0.322	-	24	3/5
4	60-80	12.9	0.497	-	24	5/5
5	Control	-	-	PBS	120	0/5

Ion-exchange chromatography

This technique was used to purify exotoxin A after precipitation with ammonium sulphate and dialysis. Column was washed several time with Tris – OH buffer (0.01) M, PH (8). Toxin were applied to column then eluted first with the Tris– OH (0.01) M, then with gradient concentration of Sodium chloride ranged between (0.1 to 0.5) M as shown in figure (2). Results showed that there are two protein peaks will appeared after elution by gradient concentration of sodium chloride, then measuring the absorbance at 280 nm. The protein fraction in the first peaks from 59 to 63 and in second peaks from 67 to 70 were concentrated by lyophilization to detect their toxicity by intraperitoneally injected to mice . Both of protein in two peaks were toxic to mice, so collection the protein in two peaks and concentrated.

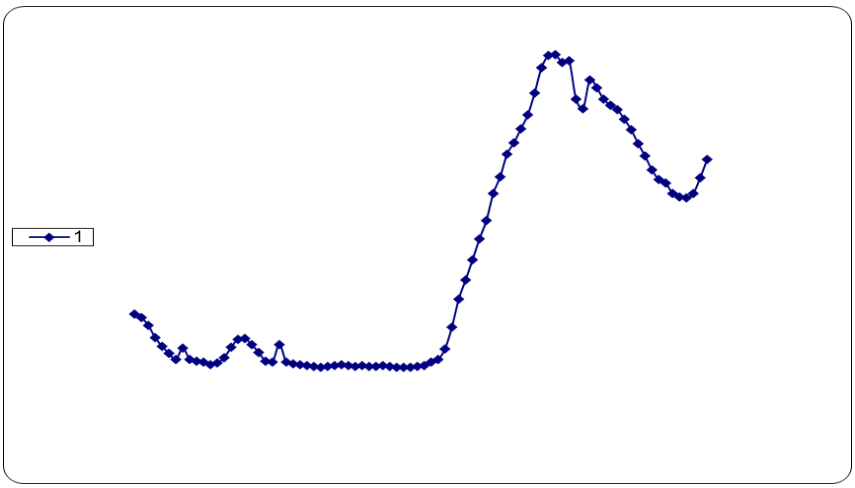

Figure 2:Ion-exchange chromatography profilof 3 ml of toxin A . DEAE-Cellulose column (1.5x35 cm) was used. The column was equilibrated with 0.1M Tris-OH pH8.0. Flow rate was 30 ml/hrs fractions of 3ml were collected.

Gel filtration chromatography

Gel filtration chromatography technique was the next step in the purification of exotoxin A after purifies it by ion exchange chromatography. Fraction that represent the toxin were collected, pooled and lyophilized for applying to Sepharose 6B previously equilibrated with (0.2) M Tris – OH buffer, PH (8) . A volume of 5 ml of concentrated toxin was applied to the column and eluted with the same buffer. results indicated in figure (3) showed that two peaks were result from elution of toxin , the protein in both fraction were pooled and concentrated then injected intraperitoneally to mice ,only beak 2 showed toxicity to mice while peak 1 are not toxic to mice when injected intraperitoeally.

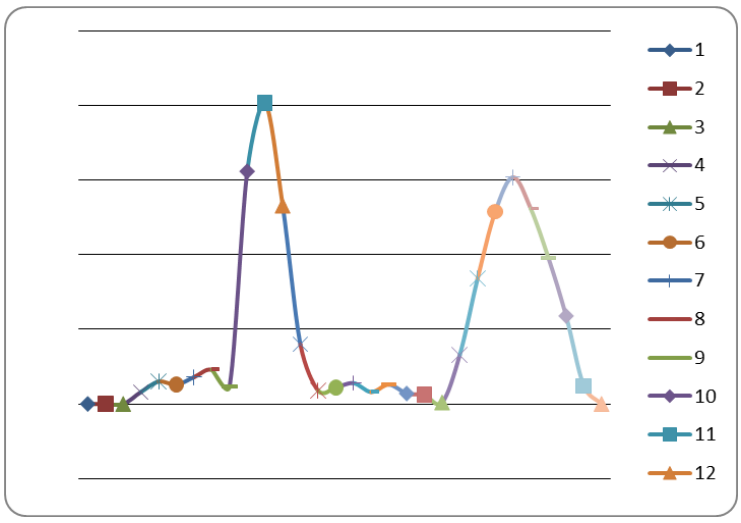

Figure-3 Represent purification of toxin A by gel filtration chromatography

Detection of molecular weight of toxin

Molecular weight of purified exotoxin A produced by pseudomonas aeruginosa (26A)Strain was determined by gel filtration using Sepharose 6B in the presence of four standard protein (Aldolase , Bovine serum albumin, ovalbumin, chytrypsin).Blue dextran 2000 was applied first to column then elution of dye void volume V0 was detected. Exotoxin A and each of standard protein were applied to the column and eluted individually then Ve of each standard protein were detected then record Ve/ Vo for each one, draw curve of molecular weight between Ve/ Vo and logarithm of molecular weight. Results indicated that toxin A has molecular weight (65.12) as shown in figure(4)

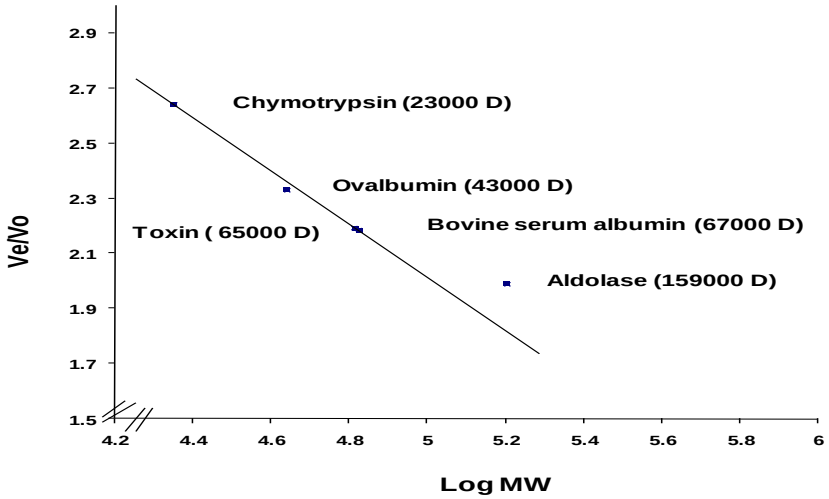

Figure- Represent standard curve for determining the molecular weight of purification of toxin A

DISSCUSSION

Identification of bacterial isolates

On gram stain the bacteria appear as gram negative rod shape straight or slightly curved, non-spore forming ,this is agreement with (Kenneth, 2011).

Morphological characteristics of the suspected isolates were done depending on the colonial shape that it form colorless colonies when cultured on the MaConkey agar due to non-lactose fermentation, also sub cultured on Cetrimide agar (N-cetyl-NNN-trimethylammonium bromide) is added to inhibit bacteria other than *Pseudomonas aeruginosa*. Its action as a quaternary ammonium cationic detergent causes nitrogen and phosphorous to be released from bacterial cells other than *Pseudomonas aeruginosa*
(Ryan and Ray, 2004) .

Isolates were subject first to number of biochemical tests, then to API 20 system and reveled that only 123 of 161will give the typical biochemical tests related to belong to *pseudomonas aeruginosa* spp

Protease test

From total of 123 isolate that related to *Pseudomonas aeruginosa spp* 97.57% strains give positive results for protease and only 3 strains (2.47%) give negative results for protease when streaked on skim milk agar ,this is in agreement with (Alane *et al* .,2009) who reported that more than 95% of pseudomonas aeruginosa isolates were positive for protease and exotoxin A. protease negative strain were used for production of toxin A because the exotoxin is ready destroyed by the protease produced by most strains of *Pseudomonas aeruginosa*, it's in agreement with (shahanra *et. al.*, 2007) who reported that Exotoxin A has been purified from culture supernatants of a nonproteolytic *P. aeruginosa* strain, PA 103, by several physicochemical methods . Also several lines of indirect evidence suggest that in cultures of *P. aeruginosa*, toxin is inactivated by proteolytic enzymes. (Mohsen *et al.*, 2004).

The three negative strains for protease were injected in mice to detect toxicity, then (26A) strain were choice because it give high killing rate compared with the others strains, this is in agreement with (Jens *et al*.,1998) that use strains devoid of protease for production of toxin then inject the stage I toxin intra peritoneally in mice to detect toxicity.

Production of pigment

For detection the ability of pseudomonas aerugiosa to produce pigment, it cultured on cetrimide agar to produce pyocyanin (a blue green, water soluble, non-fluorescent, phenazine pigment) which is stimulated by the inclusion of magnesium chloride and potassium sulfate in the broth. Cetrimide (N-cetyl-NNN-trimethylammonium bromide) is added to inhibit bacteria other than *Pseudomonas aeruginosa*. Its action as a quaternary ammonium cationic detergent causes nitrogen and phosphorous to be released from bacterial cells other than *Pseudomonas aeruginosa* (Kennth , 2011)

Production of toxin A

Production of toxin A from *Pseudomonas aeruginosa* strain (26) A on tryptic soy dialysate enriched with 1% glycerol and mono sodium glutamate (1) M in shaker at 32c for 24 hr will increase the production of toxin, the growth and toxin production will enhanced by shaking and aeration, also change the concentration of monosodium glutamate from 0.05 to 1 M will increased production of toxin this in agreement with(Guadarrama *et al* ., 2005) who reported that he maxium production of toxin A in tryptic soy dialysate with of addition 1% glycerol and 1M monosodium glutamate to the medium in shaking rather than in static condition.

Zinc acetate precipitation

Zinc acetate salts are used for precipitation of stage I toxin toxin A ,incubation of toxin with (1/20 volume) of 1 M zinc acetate at 4c for 2-3 hours and for 24 hr .results show that precipitation of toxin will show at both times but incubation of zinc acetate at same concentration but longer period 24 hours rather than 2-3 hr will increased the recipitation of toxin A and increased toxicity to mice when injected intraperitoneally. This in agreement with(Stephen *et al*.,1988) while (Robert,1979) precipitate toxin A by 1 M of zinc acetate at 4c for few hours then detection their toxicity in mice.

Ammonium sulphate precipitation

Ammonium sulphate is the common salts used in protein precipitation due to high solubility, stabilizing protein structure, as well as cheapness so it frequently used for concentration of protein A.

Our results show that of stage I toxin saturation with (60 – 80) % ammonium sulphate will give higher concentration of stage II toxin A and higher toxicity in mice compared to other groups .this is agree with (Ali et al., 2009) who used 70% of ammonium sulphate. (Liu, 1973) record that saturation of stage I toxin with 60% ammonium sulphate will precipitate more than 75 % that will be toxic in mice when injected intraperitoneally.

Ion-exchange chromatography

Results showed that the two protein peaks that appeared after elution of toxin by Tris-OH buffer then by gradient concentration of sodium chloride and measuring the absorbance at 280 nm. The protein fraction concentrated by lyophilization then injected intraperitoneally injected to mice to detect their toxicity. Proteins in both peaks were toxic to mice, so collection the protein in both peaks and concentrated. This in agreement with (Michael *et al.*,1977) who reported that toxin peaks results from Ion-exchange chromatography eluton first by 0.01M Tris – OH buffer ,pH 8, then by gradient concentration of NaCl will be toxic to mice when inject intra peitoneally.

Gel filtration chromatography

Fraction that represent the toxin were collected, pooled and lyophilized for applying to Sepharose 6B previously equilibrated with (0.2) M Tris – OH buffer, pH (8) . results showed that two peaks are result from elution of toxin , the protein in both fraction were pooled and concentrated then injected intraperitoneally to mice ,only beak 2 showed toxicity to mice while peak 1 are not toxic to mice when injected intraperitoeally. This in agreement with (Guadarrama *et al* ., 2005) who reported that large peak 1 that represent nontoxic protein preceded peak 2 that contained the toxin and were toxic to mice when injected intrapeitoneally ,while the peak 2 are not toxic.

Molecular weight of purified toxin A

The molecular weight of purified toxin A that applied to gel filteration column and eluted, then returned to curve of standard protein elution was estimated to be 65.12 and this in agreement with (Boudenet *et al*., 2004) who noted that exotoxin A, a 66,000-dalton polypeptide, is the most toxic component of these virulence factors also in agreement with (Stephen *et. al*., 1988; Melanine *et. al.,* 2006). While disagree with (Michel *et. al*., 1977) who reported that PA toxin is produced as a single polypeptide chain with a molecular weight of about 71,500.

REFERENCES

Alane B .V., Maria N.L., Anade L., Simone D. G. and Marta H .B. (2009). Detection of extra cellular protease from microorganism on agar plate. Department of general microbiology, Fedral University, Rio –de Janero,Brasil.

Ali M., Jamshid K., Davood M., Aziz J. and Nazanin K. (2009). Active immunization using exotoxin A confers protection against *pseudomonas aeruginosa* infection in mouse burn model. *BMC. Microbiol.* 9 :1-10.

Aufaugh E., William G. C. and Clifford S. (2010). Glycolipid dependent protease sensitive internalization of *pseudomonas aeruginosa* in to cultured human respiratory epithelial cell. *J. Microbiol.* 4 :106-115.

Boudenet S., Doyonnas R., Vacdanheron M.J . and Cozzone A.J. (2004) . The cytotoxicity of *pseudomonas* exotoxin A, inactivated by modification of the cell-binding domin I is restored when conjucated to an erythroid cell specific targeting agent, cellular oncology group ,division of biological science. *Nat. RES. Coun. Canada.*

Bradford M. (1976). A rapid and sensitive method for the quantitation of microgram quantites of protein utilizing the principle of protein –dye binding. *Annu. Biochem.* 72:248-254.

Chotirmall S. H.,, Smith S.G. and Guanaratnam C. (2012). Effect of estrogen on *pseudomonas* mucoidy and exacerbation in cystic fibrosis. *Eng. J. Med.* 336:1978-1988

Guadarrama S., Palcini E.D ., Broadaway S. C. and Pyle B. H. (2005). *Pseudomonas aeruginosa* growth and production of exotoxin A in astatic and Modeled microgravity environment. Gravit. Space. Biol. 18(2) :85 -88

Ira P. (2003). Immunotoxin containing *pseudomonas* exotoxin A: ashort history. *Cancer. Immunother*. 52:338-341.

Jens S., Sabine A., Renate B., Michael L. and Gisa T. (1998). Acute hepatotoxicity of *pseudomonas aeruginosa* exotoxin A in mice depend on T cell and TNF. *J. Immunol.* 161:5745-5754.

Jorgensen R., Merril A .R., Yates S .P and Bonsen T. (2005). The nature and character of the transition state for ADP ribosyl transferase of *pseudomonas aeruginosa*. *Nature.* 18 :979- 984 .

Josphen E.W., Christen B .T., Magdalena D.P., Tanya M. R. and David B.M .(2001). Refined crystallographic structure of *pseudomonas aeruginosa* exotoxin A and its Implication for the molecular mechanism of toxicity. *J. Mol. Biol.* 314: 823 -837.

Judith J.F., Michel R .T., Randal E. M. and Catherine B.S. (1991). Mouse liver contains *pseudomonas aeruginosa* exotoxin A-binding protein. *Infect. Immun.* 59(9): 2880- 2884 .

Kenneth T. (2011). Pseudomonas aeruginosa Rev. Todar oline text book of bacteriology.

Liu V. L. (1973). Exotoxin of *pseudomonas aeruginosa*,factors that influence the production of toxin A. *J. Infect. Dis*. 128(4):506-510.

Melanine J .F., Filiatrault K .F., Helen N .and Barbara H.I . (2006). Identification of *pseudomonas aeruginosa* genes involved in virulence and anaerobic growth. *J. Infect. Immun.* 74(7): 4237-4245.

Meluleni G. J., Grout M., Evans D. J. and Pier G.B. (1995). Mucoid *pseudomonas aeruginosa* growing in abiofilm in vitro are killed by opsonic antibodies. *J. Immunol.*155:

Mohsen F. N . and Dileep D. (2004) . Potential application of protease isolated from pseudomonas aeruginosa DP100. *J. Biotech.* 8 :1-6 .

Reuter S., Sigga A., Wiedeck H. and Trautmann M. (2002). Analysis of transmission pathways of *pseudomonas aeruginosa* between patients and tap water outlets. *Crit. Care. Med.* 30 (10):2222- 2228 .

Robert M.D. (1979). Production of exotoxin A by *pseudomonas aeruginosa* in chemically defined media. *Infect. Immun.* **24**(1):132-138.

Ryan K .J. and Ray G. (2004). Sherris medical microbiology.4th(ed).McGraw Hill. New York.

Shahanara B., IfTikhar A A., Faisal A. and Jalaluddin A. H. (2007). Chacterization of intracellular protease from *pseudomonas aeruginosa*. *PAK .J. Med. Sci.* 23(2): 227-232.

Stephen L., Mark S.S. and Kit J. (1988). Expression and secreation of the cloned *pseudomonas aeruginosa* exotoxin A by Escherichia coli. *J. Bacteriol.* **70**(2):714-719.

Susan P.Y., Patricia L. T. and Rod M. (2005). Structure function analysis of the catalytic domin of exotoxin A from *pseudomonas aeruginosa*. *J. Biochem.* 385: 667- 675.

