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Effect of Exotoxin a Isolated from *Pseudomonas Aeruginosa* Against Human Cancer Cell Lines

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Abstract

In This study, the presence of *Pseudomonas aeruginosa* was investigated from many clinical samples, and then about 54 isolates were obtained, diagnosed with all different diagnostic methods, and then the bacterial isolates produced for Exotoxin A were detected by the ELISA method and then the most productive isolate was chosen for purification by means of Precipitation with ammonium sulphate and ion-exchange column and detection of molecular weight was (M.W 65.03KD).The competent isolation tested its toxicity against two cancer cell lines HeLa and PC3 which were an inhibition ratio 69.1% and 61.6% respectively in high concentration.

***Key words**: p. aeruginosa , exotoxin A, chromatography ,cancer cell line*

Introduction

Pseudomonas aeruginosa is considered an important opportunistic pathogen[1, 2]. Causing dangerous infections, especially in immunocompromised patients[3]. This infection is difficult to treat due to the great resistance it possesses [4]. In addition; it has a great ability to resist a wide range of antimicrobial agents. Exotoxin A (ETA) is regarded as the important virulence factor secreted by *P. aeruginosa*. Liu was 1st who disgonized and purified it [5, 6] . That ETA is considering an ADP-ribosylating toxin which works in the inhibition of protein synthesis and finally cell death [7] . ETA is a single chain polypeptide of a MW 66-kDa. It is binding to receptors as specific via endocytosis receptor-mediated [8].

Material and Methods

Collection of Samples

This study have collection about 73 samples from pathogenic cases as Burns, Wounds, UTI infection, Otitis media, in AL-Ramadi Teaching Hospital and AL-Fallujah Teaching Hospital, throughout the period from 1/7/2020 to 20/9/2020. Samples were obtained by utilizing sterile cotton swaps whereas sterile UTI infection was taken by sterile container. Samples were streaked directly on agar of MacConcky and incubation was done for 24 at 37 °C.

P. aeruginosa Isolation and Identification

For the purpose of isolating and diagnosing bacteria, several tests were performed:

Morphological examination: Morphological examination was performed with Gram stain and spore-forming stain by light microscopy

Culture examination: Culture examination was performed by cultured bacterial colonies on the selective media MacConkey agar, cetrimide agar and incubation was done for 24 at 37 °C.

Biochemical exams: many biochemical exams were conducted for diagnosis bacteria including: IMVC, urease, triple sugar iron, oxidase and catalase tests for diagnosing confirmed of the isolates were performed vitek-2 system.

Production of pyocyanine: The bacteria were

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cultured on agar of cetrimide and incubation was done for 24 at 37 °C, as a result, the green color presence indicates +ve results.

Growth ability at 42 °C: The bacteria were cultured on agar plates and incubation was done for 24 at 42 °C, bacterial growth at such temperature mentions +ve results.

Protease test: For the detection the ability of bacteria to produce protease enzyme that were streaked on skim milk agar.

Detection of ETA: For the detection the ability of bacteria to produce Exotoxin A were tested using ELISA kit.

Partial Purification of ETA

Preciptation with $(NH_4)_2SO_4$: the procedure adopted in this study was performed according to [9] . Toxin was precipitated by addition of $(NH_4)_2SO_4$ at (20, 30, 40, 50, 60, 70, 80) % saturation. The product of 80% saturation that precipitated was utilized for obtaining complete toxin precipitation, and centrifugation for 30 min at 10,000 rpm was done for separating the precipitant.

Ion exchange chromatography Purification: Such was performed based on [10] through using column of DEAE-cellulose 2.5 x 15 cm that several times was washed with buffer of equilibration 0.01 M Tris-OH of pH 8.

Toxin MW determination: gel filtration chromatography was used for toxin MW purified partial determination, toxin A was kept to flow via a glass column (1.5 x 60) cm which packed with Sepharose 6B, and then in a flow rate eluted (3 ml /fraction). Trypsin 23KDa, ovalbumin 43KDa and bovine serum albumin (BSA) 67 KDa were protein standards.

Protein concentration determination: concentration of protein was performed based on [11] as following: A standard curve of BSA was carried out by utilizing various concentrations from stock solution of BSA based on volumes.

Cell lines and growth conditions: Cell lines of cervical cancer (HeLa), prostate cancer (PC-3), and normal ones were utilized for determining the ETA effect. Such cells were on media of MEM and RPMI were cultured that were enriched with mixtures of FBS (10%) and penicillin streptomycin (1%). Incubation was done at 5% CO2 and 37 °C [12] .

MTT cytotoxicity assay: Based on [13] Exotoxin A cytotoxicity was performed.

Statistical Analysis

The data that obtained were displayed as mean ± SD and statistical significances were measured utilizing test of ANOVA [14 [.

Results and Discussion

The Results of this study refer to clinical samples as total of 73 were taken from various cases as showed in (Table1). About only 54 isolates (71.05 %) gave morphological features and biochemical tests in relation to *P. aeruginosa* , while the remaining isolates of 19 might related to other bacteria being pathogenic from various genera as illustrated in (Figures 1,2). Diagnosis of the bacterium was done by means of Gram stain, and looks as non-sporulating, slightly or a straight curved, motile G- rod that aerobically growing, this is agreement with [15] . Generally, once clinical samples are cultured on agar of MaConkey, it showed all the essential traits of growing on that medium. The isolates cultured on agar of blood and appear as β hemolytic. Morphological suspected isolates features were pereformed based on the shape of colonies which if formed as colorless if cultured on agar of MaConkey because of no fermentation of lactose. Also, sub-culturing on agar of Cetrimide was added for bacterial inhibition other than *P. aeruginosa* [16] . According to the results of biochemical tests that revealed that only 54 isolates were belong to *P. aeruginosa*. These isolates gave +ve results for tests of catalase, oxidase, citrate utilization, while -ve results were obtained in production of indol and test of methyl red- voges proskour.

Colonies cultured on nutrient agar for 24 h at 42^0C can grow normally; where growth appearance mentions +ve results. also All the bacterial isolates are represented *P. aeruginosa*, and were positive for protease this is in agreement with [17] who revealed that isolates of *p. aeruginosa* as over than 95% were +ve for Exotoxin A and protease. For the purpose of pigment production from such bacteria, it cultured on agar of cetrimide for

production of pyocyanin (H_20 soluble, a blue green, non-fluorescent, phenazine pigment) that is stimulated by the inclusion of $MgCl_2$, and K_2SO_4 in the broth. Cetrimide was added to inhibit other bacteria. Its function as cationic detergent being quaternary ammonium leads to release of N and P from cells of bacteria other than *P. aeruginosa* [16] .

Exotoxin A Detection

For exotoxin A detection produced by *P. aeruginosa* isolates, kit of ELISA was utilized. About of 54 isolates, only 21 were detected of exotoxin A production. For concentrations measurement, the same kit was utilized also as the displayed in (Table 2). Isolates screening mentions that isolates mostly show +ve results. Nevertheless, only one isolate was elected based on their productivity being the highest besides their distribution in various sites when infections, highest isolates called *P. aeruginosa* 15, which produce (27.56) ng/ml and distributed in burn cases.

Purification of Exotoxin A: such steps are includes:

Proteins precipitation by $(NH_4)_2SO_4$: for toxin crude extract concentration and H_20 removing as abundant as possible, $(NH_4)_2SO_4$ was utilized at (20, 30, 40, 50, 60, 70, and 80) % saturation. For exotoxin A precipitation, the ratio of saturation as 80% was elected. Such step permits the molecules salting out of from H_20. Since $(NH_4)_2SO_4$ able to neutralize charges at the protein surface and disrupting layer of H_20 contiguous the protein, it will cause eventually a decline in the protein solubility that in turn cause protein precipitation via the salt effect [18,19] .

Exotoxin A partial purification via ion-exchange chromatography: Exotoxin A partial purification via ion-exchange chromatography was done by DEAE-cellulose utilizing. Figure 3 illustrated the DEAE-cellulose column wash and elution to select isolate. In the wash steps, exotoxin A was detected, whereas the fractions as eluted were revealed. The obtained results revealed the existence of two peaks. Nevertheless, just one peak for each select isolates elution exposes activity as found via kit of ELISA. The purified partial proteins amounts mention as much as protein of 0.053 mg/ml produced via isolates PA15, the number fraction was between 35-37. Results revealed that there are one protein peak will seemed following elution by gradient NaCl concentration, and then the absorbance was measured at 280 nm.

Toxin M.W detection

The M.W was assigned to ETA produced by *P. aeruginosa* was measured via gel filtration utilizing Sepharose 6B in the existence of three proteins as standards (BSA, ovalbumin, trypsin). Every standard of protein and Exotoxin A were column-applied and individually eluted and then Ve of every standard protein were measured then recorded Ve/Vo for every one. The Results indicate that ETA has M.W (65.03) as illustrated in Figure 4.

Assay of Cell viability

Effect of cytotoxicity was done by using technique of MTT. Figure 4 showed that declining in cells viability of PC3 and HeLa by concentrations and incubation period increasing of the extracts was noticed. Significant inhibition against HeLa ($P<0.05$) in most concentrations and incubation periods was observed. ETA extract of (400 µg/mL) as the highest concentration caused effect in maximum time as highest inhibition against PC3. High effect as cytotoxic against Hela and PC3 was noticed if with ETA extract were treated which were 61.6% and 69.1%, respectively. However, they were of week activity versus cell line being normal. Normal cells were growing well in around 91%. These results might be explaned due to cancer cells death that occurred by apoptosis that is recognized as a controlled event. The cytokines production are known to be as anti-inflammatory molecules besides phagocytosis can cause this type of cell damage [20] . Consequently, it was believed that the ETA was of biological activity that might inhibit proliferation of cancer cells [21] .

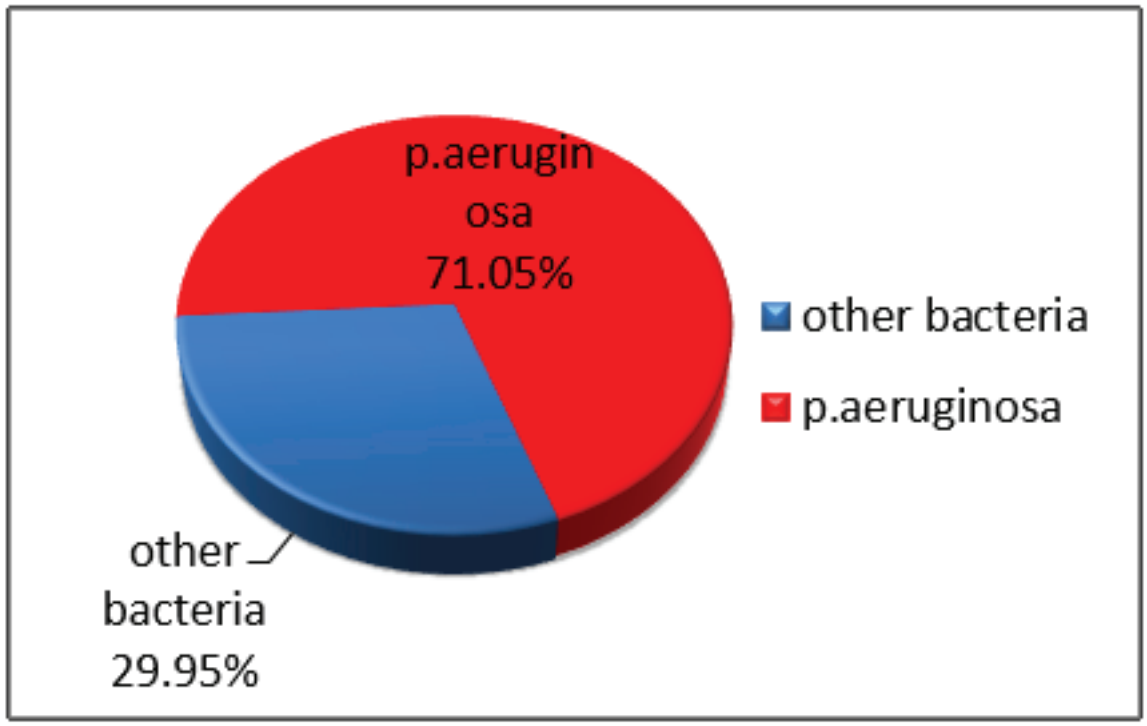

Figure (1): percentage of isolated *P.aeruginosa*

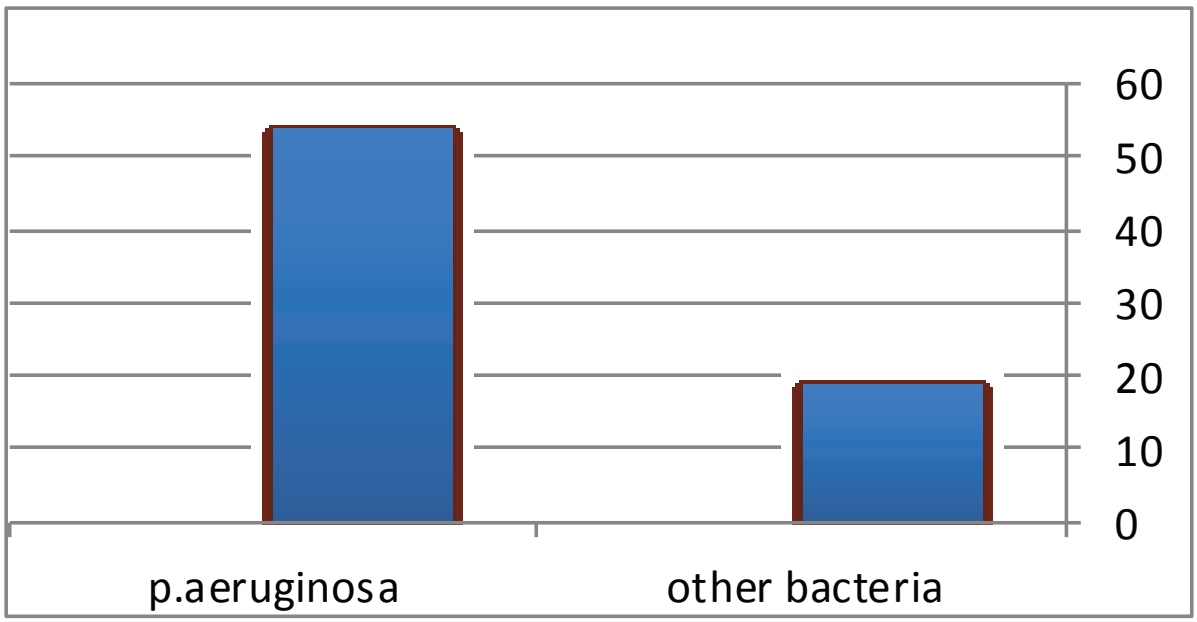

Figure (2): Number of the isolates *P.aeruginosa*

Table(1) Source and number of *P.aeruginosa*

Sources	No. of samples	No .positive isolates
Burns	36	30
Wound	14	11
Otitis media	14	8
UTI	12	5
Total	76	54

Table (2) The concentration of ExotoxinA isolated from *P.aeruginosa* isolates

NO	NO. isolates of produced ETA	Specimen	Concentration toxin ng/ml
1	8	UTI	15.22
2	9	Ear	11.12
3	13	Ear	11.45
4	15	wound	27.56
5	19	wound	21.34
6	20	wound	22.11
7	24	UTI	9.23
8	29	sputum	9.77
9	33	wound	22.76
10	34	UTI	19.88
11	35	sputum	8.65
12	36	Burn	18.11
13	37	wound	24.34
14	38	wound	13.66
15	39	UTI	17.65
16	44	wound	23.88
17	45	burn	17.45
18	47	UTI	16.88
19	49	wound	25.45
20	51	wound	22.32
21	52	burn	21.66

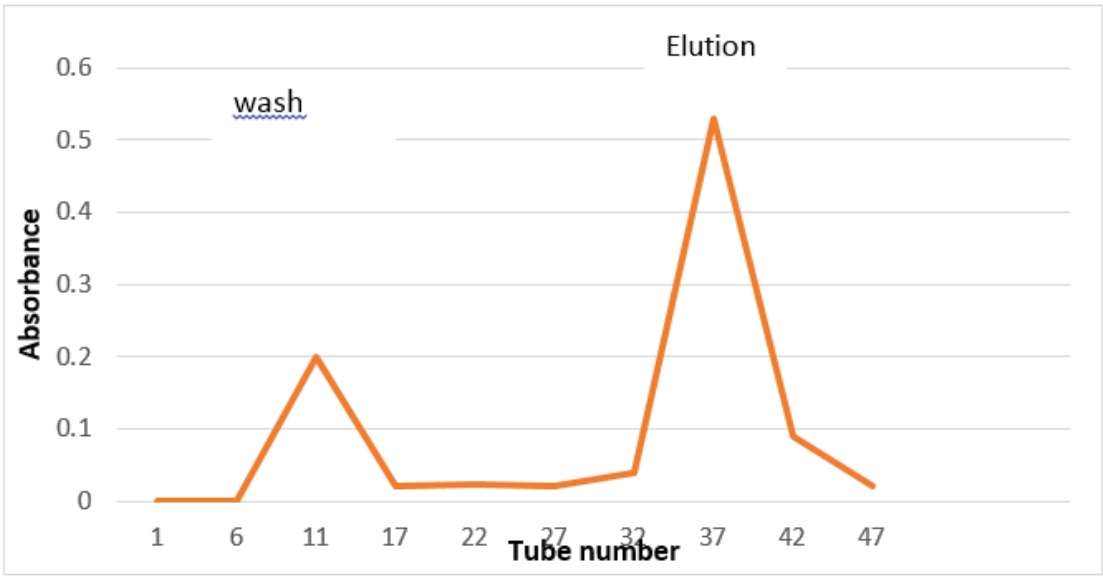

Figure (3):purification of exotoxinA produced from PA15 isolate by DEAE-cellulose ion exchange chromatography column (2.5x15)equilibrated with 0.01m Tris HCl

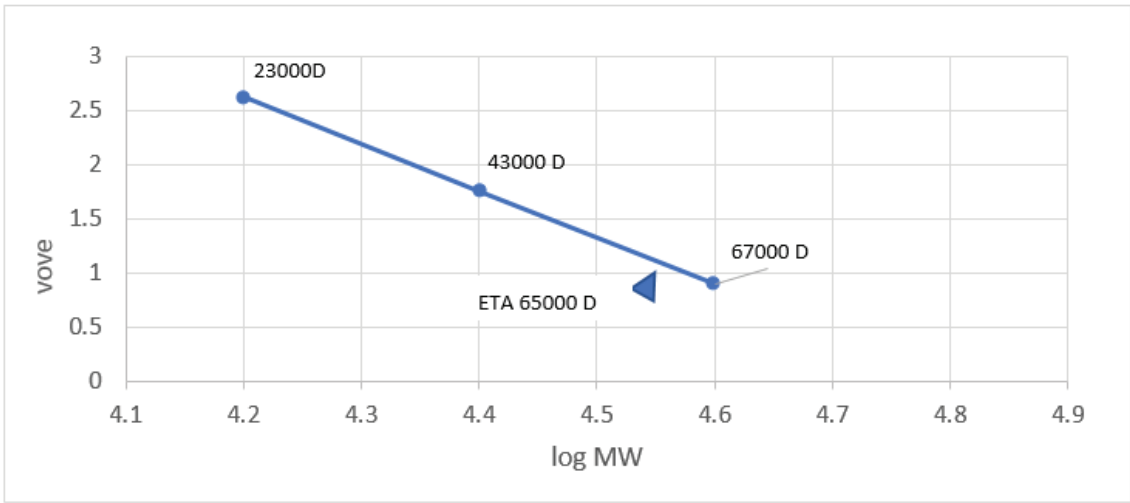

Figure (4):Measured molecular weight of Exotoxin A according to ratio to ve/vo

Conclusion

This study was conducted on extracted and purification of exotoxin A produced by local strains of *P. aeruginosa* in Iraq then detection the molecular weight of toxin and have cytotoxicity against cancer cell lines.

Conflict of Interest: No conflict of interest

Funding: Self ,

Ethical Clearance: This study is ethically approved by the Institutional ethical Committee.

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