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Study the Effect of Locally Produced *Clostridium perfringens* Enterotoxin on the MCF-7 and T47D Breast Cancer Cell Lines

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Abstract

Breast cancer is the most common cancer among women worldwide after skin cancer. It is also the second leading cause of cancer death in women after lung cancer .One in eight women in the United States (12%) will develop breast cancer in her lifetime and this percent is in a continuous increasing .Breast cancer treatment regimens differ widely based on the type of cancer, its stage, its sensitivity to hormones, the patient's age and health, and other factors. Surgery and radiation therapy are mainstays of breast cancer treatment as well as chemotherapy. Despite of the remarkable development in cancer treatment and new drugs ,but the progress in recovery of cancer still weak so the need to new strategies of therapy and to new drugs remains very important . One of these strategies is the gene therapy by which such strange toxic molecules specifically are directed to the cancer cells ,one of these candidates that attracting attention are the bacterial toxins which revealed high activity in killing of the cancer cells in in vitro and in vivo studies . Our study paid attention to one of the bacterial toxins to screen on its activity in this respect . In more details, we screen the activity of locally isolated Clostridium Perfringens enterotoxin on the breast cancer cell lines as a first step and for the first time locally to detect on the effect of bacterial toxins isolated from our environments as well as there is no previous local study on CPE that extracted and purified from bacteria isolated from food poisoning patients in Mosul city.

Keywords: Clostridium perfringens enterotoxin; toxin; cancer; Claudin 3; MCF-7; T47D.

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1. Introduction

Clostridium perfingeus has been described as a prominent pathogen for humans and livestock and a cause of both tissue-toxic diseases and Intestinal diseases, which are known as enteritis. The studies indicated that approximately 5% of *C.perfingeus* isolates produce a enterotoxin called CPE (*Clostridium perfingeus* enterotoxin), and most strains positive for the production of CPE toxin are described as type A (*C.perfingens* type A). CPE is responsible for causing food poisoning, which is the second most important cause of food poisoning in most developing countries [1].

CPE toxin is produced during the spore formation phase in the intestine. It is a single polypeptide containing 319 amino acids containing initial unique sequence. The action of CPE begins with its association with Claudin, specially Claudin 3 and Claudin 4, although more than 18 Claudin proteins have been diagnosed, only these two proteins are present on cells sensitive to the degradation resulting from the CEP toxin, binding occurs between binding units of the carboxylation end with a special part in the second cell extracellular loop of Claudin proteins [2].The highly expression of Claudin 3 and 4 were done on human cancers such as prostate, breast, pancreatic and ovarian cancers, This may provide an important molecular tool to target human cancer cells with specialized technology. It was found that high expression of Claudin 3 and Claudin 4 receptors occurred by 62% and 26%, respectively in breast cancer. Breast cancer is considered to be the second most important cause of death among other types of tumors, and in response to these advantages, a trend has been made to develop methods based on the use of toxin in the diagnosis and treatment of breast cancer [3], In this study, for the first time locally, the biological activity of CPE toxin against breast cancer cell lines including MCF-7 and T47D was investigated, as well as its effectiveness against normal cells such as periodontal fibroblast cells and Vero cells (hepatocytes).

2. Materials and methods

2.1. Clostridium perfringens enterotoxin A

Clostridium perfringens enterotoxin A protein isolated from locally *C.perfringens* type A isolate.

2.2. Cell lines and cell culture

Different cell lines have been used in this study including the human breast adenocarcinoma cell lines MCF7 and T47D, peridonntal fibroblasts and the Vero cell line were obtained from laboratory of molecular biology research in faculty of medicine/university of Jordan.The MCF7 and T47D and fibroblasts cell lines were cultured in DMEM/F12 medium (Gibco, invitrogen, USA). the Vero cell line was cultured in RPMI 1644.Media were supplemented with 10 % heat inactivated fetal bovine serum (FBS)(Gibco, invitrogen, USA), 1% of 2 mM L-glutamine (Lonza, Belgium),50 IU/ml penicillin (Lonza,Belgium) and 50 µg/ml streptomycin (Lonza, Belgium). All cells were maintained at 37ºC,5%CO_2 humidified incubator.

2.3. In vitro antiproliferative and cytotoxicity assay using trypan blue assay

MCF-7,T47D and Vero cell were seeded at density of 1.0 $\times 10^6$ cell/well. Fibroblast cells were seeded at 0.35

$\times 10^6$ cell/well. All cell line were cultured in 6- well plate. Afterwards, cells were treated with *Clostridium perfringens* enterotoxin A at five different concentrations(1.00,0.50,0.25,0.13 and 0.06)µg /ml. Each concentration was added to the plate in three replicates. Control wells contained media only. After 1 h incubation, cell viability was determined by trypan blue assay. Dead cells and live cells were counted [4].

2.4. In vitro antiproliferative and cytotoxicity assay using MTT assay

Cells were seeded at density of 10.000 cell/well in 96-well plate. Afterwards ,cells were treated with enterotoxin at six different concentration (1.00, 0.5, 0.25, 0.125, 0.062 and 0.00312) µg/ml.each concentration was added to the plate in three replicates. control wells contained media only. after 1 h incubation, cell viability was determined by MTT assay according to cell proliferation assay kit(promega, USA).absorbance(OD) was measured at 570 nm with background subtraction at 630. [5].

2.5. Statistical Analysis

The percentage cell viability was calculated with respect to control as follows:

% Cell viabilility =(absorbance of test÷ absorbance of control)×100

The IC_{50} is half the maximal inhibitory concentration of the toxic compound which results in the reduction of biological activity by 50%. IC_{50} was determined.

3. Result

The result of antiproleferative and cytotoxicity assay of CPE against breast cancer cell line (MCF-7 and T47D),liver cell line(Vero cell) as positive control and normal cell (fibroblast cell) by using trypan blue assay are shown in (table 1). The result showed that the concentration of MCF-7 and T47D in addition Vero cell decrease when the increase the concentration of CPE in a dose dependent fashion beginning with the concentration 0,06 represent the less inhibition finishing with the high toxicity at 1 µg/ml concentration with $0.04\times10^{6,}$ 0.03×10^6 and 0.25×10^6 while no inhibition seen for fibroblasts cell at any concentration. A viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm (figure1).

Table 1: In vitro antiproleferative and cytotoxicity assay using trypan blue assay.

Cell line	Concentration(µg/ml)					
	control	0.06	0.13	0.25	0.50	1.00
MCF-7	1.00×10^6	0.75×10^6	$o.33\times10^6$	$o.23\times10^6$	0.10×10^6	0.04×10^6
T47D	1.00×10^6	0.35×10^6	0.24×10^6	0.15×10^6	0.08×10^6	0.03×10^6
Fibroblast	0.35×10^6	0.35×10^6	0.35×10^6	0.35×10^6	0.35×10^6	0.35×10^6
Vero	1.00×10^6	0.9×10^6	0.82×10^6	0.45×10^6	0.30×10^6	0.25×10^6

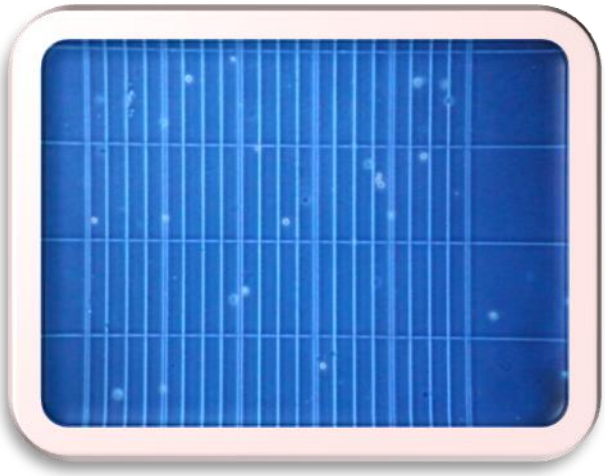

Figure1: Cell line stained by trypan blue stain.

Result of cell growth inhibition by the CPE against MCF-7,T47D,Vero cell and fibroblast cells by using MTT assay depending on the absorbance are shown in (tables2,3,4 and 5).the result depending on the absorbance were indicated that the absorbance increased when the concentration of CPE decreased in the high concentration 1.00 µg /cm^3,the absorbance 0.106 while reached 0.617 at 0.00313 µg /cm^3 for replicate 1.the same effect was observed in the other two replicate.

Table 2: The absorbance of **MCF-7** treatment by CPE.

Control	Concentration(µg/ml)					
	1.00	0.5	0.25	0.125	0.062	0.00312
0.638	0.106	0.19	0.203	0.382	0.564	0.617
0.577	0.069	0.094	0.191	0.373	0.56	0.5
0.581	0.113	0.088	0.157	0.424	0.51	0.555

Table 3: The absorbance of **T47D** treatment by CPE.

Control	Concentration(µg/ml)					
	1.00	0.5	0.25	0.125	0.062	0.00312
0.354	0.077	0.085	0.083	0.1	0.113	0.213
0.312	0.071	0.082	0.076	0.083	0.114	0.21
0.309	0.07	0.072	0.07	0.075	0.081	0.222

Table 4: The absorbance of **Vero cell** treatment by CPE.

Control	Concentration(µg/ml)					
	1.00	0.5	0.25	0.125	0.062	0.00312
0.364	0.155	0.23	0.29	0.349	0.346	0.44
0.413	0.188	0.238	0.302	0.328	0.348	0.363
0.377	0.165	0.197	0.357	0.379	0.355	0.35

Table 5: The absorbance of **fibroblast cell** treatment by CPE.

Control	Concentration(µg/ml)					
	1.00	0.5	0.25	0.125	0.062	0.00312
0.364	0.155	0.23	0.29	0.349	0.346	0.44
0.413	0.188	0.238	0.302	0.328	0.348	0.363
0.377	0.165	0.197	0.357	0.379	0.355	0.35

When the percentage of cell viability of MCF-7,T47D were calculated depending on the absorbance of MTT assay and compared to normal cell, significant decreased in breast cell lines as the toxin concentration increased in a dose-depending fashion. The viability of MCF-7 at lower concentration 0.00312 µg/cm^3 as 92.962%,gradually decreased with increasing concentration until it arrived 16.007% at higher concentration 1.00µg/cm$^{3.}$ The same effect had been shown for T47D and Vero cell while normal cells did not effect by CPE (table 6,7,8, 9 and 10).In general, the morphological damage of the MCF-7 breast cell lines can be observed clearly compared to normal cell that does not undergo to any change (figure 2,3 and 4).

Table 6: The percentage of cell viability of **MCF-7** by MTT assay (In vitro).

Cell viability%	Concentration(µg/ml)					
	1.00	0.5	0.25	0.125	0.062	0.00312
Replicate 1	16.614%	29.780%	31.818%	59.874%	88.401%	96.708%
Replicate 2	11.958%	16.291%	33.102%	64.644%	97.053%	86.655%
Replicate 3	19.449%	15.146%	27.022%	72.977%	87.779%	95.524%
Average	16.007%	20,405%	30.647%	65.831%	91.077%	92.962%

Table 7: The percentage of cell viability of **T47D** by MTT assay (In vitro).

Cell viability%	Concentration(µg/ml)					
	1.00	0.5	0.25	0.125	0.062	0.00312
Replicate 1	16.614%	29.780%	31.818%	59.874%	88.401%	96.708%
Replicate 2	11.958%	16.291%	33.102%	64.644%	97.053%	86.655%
Replicate 3	19.449%	15.146%	27.022%	72.977%	87.779%	95.524%
Average	16.007%	20,405%	30.647%	65.831%	91.077%	92.962%

Table 8: The percentage of cell viability of **Vero cell** by MTT assay (In vitro).

Cell viability%	Concentration(µg/ml)					
	1.00	0.5	0.25	0.125	0.062	0.00312
Replicate 1	42.520%	63.186%	79.670%	95.879%	95.054%	102.879%
Replicate 2	45.520%	57.627%	73.193%	79.418%	84.261%	87.893%
Replicate 3	43.766%	52.254%	94.694%	100.530%	94.164%	92.838%
Average	43.956%	57.689%	82.519%	91.942%	91.159%	100.536%

Table 9: The percentage of cell viability of **Fibroblast cell** by MTT assay (In vitro).

Cell viability%	Concentration(µg/ml) 1.00	0.5	0.25
Replicate 1	11.392%	116.455%	128.481%
Replicate 2	96.63%	116.455%	128.481%
Average	104.0%	116.455%	128.481%

Table10: The Average of cell viability % of cell lines by MTT assay (In vitro).

Cell line	Concentration(µg/ml) 1.00	0.5	0.25	0.125	0.062	0.00312
MCF-7	16.007%	20,405%	30.647%	65.831%	91.077%	92.962%
T47D	22.386%	24.531%	23.483%	26.373%	31.557%	66.44%
Vero cell	43.956%	57.689%	82.519%	91.942%	91.159%	100.0%
Fibroblast cell	100% viability					

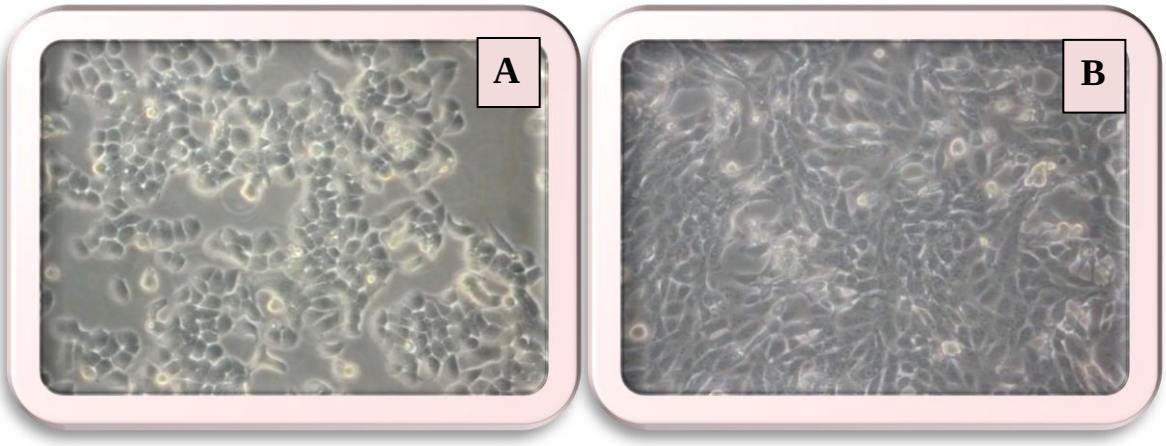

Figure 2: A: MCF-7 treatment with 0.125 µg/ml CPE, B: MCF-7 without treatment.

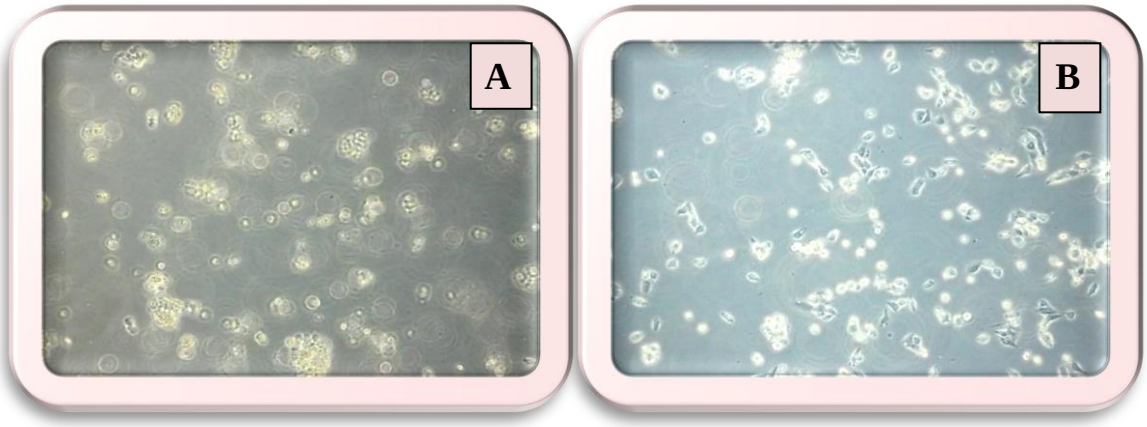

Figure 3: A: MCF-7 treatment with 1.00 µg/ml CPE, B: MCF-7 treatment with 0.5 µg/ml CPE.

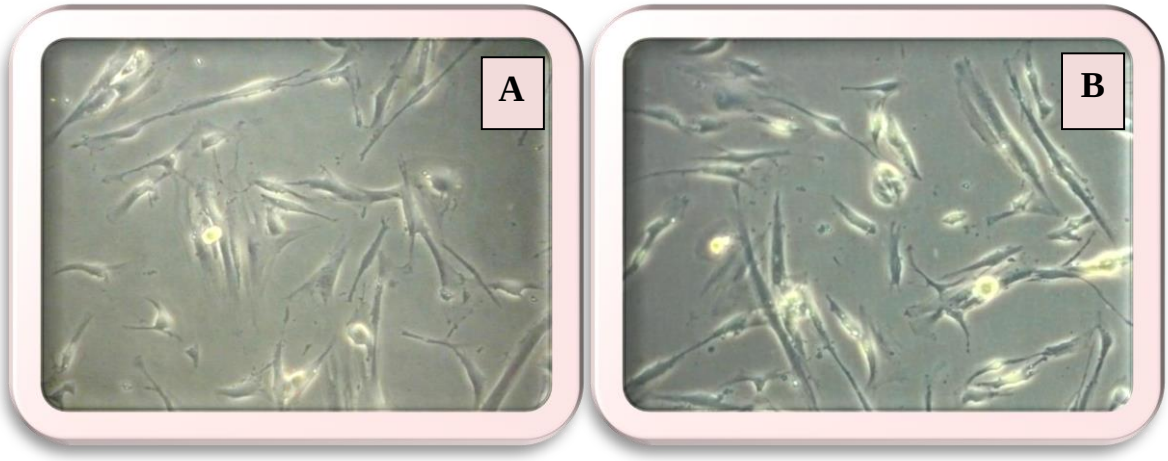

Figure 4: A: Fibroblast cell treatment with CPE, B: Fibroblast cell without treatment.

The IC_{50} value was found to be 208.27 by MTT assay for CPE,it had a high activity against MCF-7 cell line while the IC_{50} value was found to be 24.53 and 1325 for CPE against T47D and Vero cell respectively (table 11),while the fibroblast maintains there viability as 100%

Table11: The **IC_{50}** of MCF-7,T47D and Vero cell (In vitro).

Cell line	Enterotoxin IC_{50}
MCF-7	208.27 ± 31.13
T47D	24.5 3± 2.99
Vero cell	1325.73 ± 171.40

4. Discussion

As we seen the result of antiproleferative and cytotoxicity assay of CPE by using trypan blue assay against MCF-7 and T47D breast cancer cell lines compared with positive control and negative control, the concentration of bteast cancer cell lines decrease when the concentration of CPE increase in a dose dependent fashion. A viable cell appear as a bright cell but a nonviable cell have a blue color this is due to fact the live cell possess intact cell membrane that exclude certain dyes such as trypan blue, whereas dead cells did not stain. The same results were obtained using the MTT assay with a dose dependent fashion. Thus, it can be said that CPE toxin is an effective substance against breast cell lines and future studies can be developed in this direction.

The incidence of breast cancer is increasing steadily in developing countries, which is a reason for the high rate of cancer –related deaths through the world, despite the remarkable development in chemotherapy, radiation therapy and the continuous development in the modern medicines,the progress in cure rates for cancer patients remained weak and for this reason there remains an urgent need for new targets and highly effective therapeutic compounds.one of the promised strategies may target the cancer by genetically treatment including the approach by which foreign toxic particles can be specifically directed cancer cells [6]. Many bacteria were used in the past

for treatment cancer such as salmonellae that cause food poisoning at the same time and can be used in cancer therapy [7], so the focus of this study in the first time in Iraq was on the use of CPE isolated from *Clostridium perfringens* was cause food poisoning for the treatment of breast cancer to provide a new tool infighting cancer in a selective way without any damage to normal cells and opening a new direction for the possibility of using CPE-based therapy for selective targeting of breast cancer cells. from our finding, the CPE toxin had a cytotoxicity against the cell lines of breast cancer compared to normal cells that is not affected.this may be due to the ability of the toxin to bind with cancer cells through specialized receptors expressed by cancer cells while it cannot be expressed in normal cells.so this property is important in giving specificity to toxin in a manner similar to the case of food poisoning due to its ability to analyze cells in specialized and rapid method for cells expressing their own receptors.in many studies,it has been indicated that CPE toxin receptors are claudin 3 and 4,these receptors are highly expressed on breast, colon, and rectal cancers. These facts presents claudin 3 and 4 as attractive target for selective treatment of many cancers [8]. Another study also indicated that CPE toxin is the key to effective cancer treatment in humans by assessment the effectiveness's of gene therapy by target the receptors *in vitro* and in vivo by using a specialized toxin vector [9] while Baker and his group that conducted by using tissue cultures and laboratory animals as amodel,the cpe is the key for the kills the cancer cells with a high efficiency[1].

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