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Influence of *Pseudomonas aeruginosa* exotoxin A against breast cancer (MCF-7) cell line

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ABSTRACT

Out of sixty-nine different clinical samples included UTI, otitis media, wound, and burn infections, fifty samples showed bacterial growth, while the remaining were negative for bacterial growth. Only thirty-one (62%) isolates were related to *Pseudomonas aeruginosa*. Results indicated that burn infections were the highest with this bacterial colonization. Detection of the exotoxin A was conducted by using an ELISA kit. Current results revealed that, out of thirty-one *P. aeruginosa* isolates, only twenty-one were able to produce exotoxin A. The isolate (P29) was selected based on its highest productivity of this toxin, reaching 29.24 ng/ml, in addition to partial purification steps for this toxin that had been conducted. The molecular weight of the exotoxin A had been determined and appeared as 65.33 kilodaltons after being compared with standard proteins. The highest concentration of exotoxin A was 400 µg/mL. The results showed that when exotoxin A, which was partially purified, was tested on the MCF-7 cell line for 72 hours at 37°C, it significantly stopped protein production by affecting elongation factor 2 through the action of ADP-ribosyl transferase, but it did not have a noticeable effect on normal human dermal fibroblasts-neonatal (HDFn).

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Introduction

An aerobic, Gram-negative, rod-shaped, motile *Pseudomonas aeruginosa* is known to be optimally adapted to various environmental conditions (Riedel, 2019). Many acute and chronic infections that can result in a high percentage of mortality rates in a variety of hosts and organs by this opportunistic pathogen can be related to its extensive repertoire of virulence factors and complex regulatory network of intra- and inter-cellular signals that enable it to adapt, flourish, evade the defenses of the host, and contribute to successful infection and disease (Vidaillac & Chotirmall, 2021, Al Jader & Ibrahem 2022, Foulkes et al. 2022; Aboelnasr et al. 2024).

Numerous virulence factors give *P. aeruginosa* the possibility for surface adherence, dissemination after tissue damage, and nutrient supply, in addition to increased survival rate (Coggan & Wolfgang, 2012; Balasubramanian et al., 2013; Qaralleh 2024). Undoubtedly, Exotoxin A is the highly toxic virulence factor of P. aeruginosa (Michalska & Wolf, 2015). *P. aeruginosa* Exotoxin A-based immunotoxins, which harness toxin moieties targeting, are usually fusion proteins (Morgan et al., 2023).

In limited iron conditions during the stationary phase, when changes in environmental temperature occur, and in the presence of specific factors like the amino acid glutamine, Exotoxin A is secreted into the

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bacterial extracellular space (Badr et al., 2008; Fito-Boncompte et al., 2011). It causes inhibition of elongation factor-2 by ADP-ribosylation of EF-2 using NAD+, which results in cessation of polypeptide elongation; moreover, failure of protein synthesis almost always occurs, and eventually, cell death happens (Yates & Merrill, 2004).

Systems of secretion are classified into six types, including multi-toxin components type III secretion system (T3SS), pili (T4SS), and flagella (T6SS-associated), which function in host colonization, adhesion, swimming, and swarming. Ultimately, Pel, Psl, and alginate, which are exopolysaccharides, may enhance biofilm formation, whereas clearance of bacteria can be impaired (Ozer et al., 2021; Filloux, 2011).

Regulation between the host and bacterial responses is indicated to be attributable to T3SS and T6SS, including colonization, biofilm formation, apoptosis of host cells, inflammatory response, bacterial competition/interaction, and motility (Horna & Ruiz, 2021; Sana et al., 2016).

The goal of the current project is to appraise the cytotoxicity of partially purified Exotoxin A produced from *P. aeruginosa* against MCF-7 cancer cell lines.

Materials and Methods

Isolation and Identification of P. aeruginosa

Different samples, including those from urinary tract infections (UTI), wound, burn, and otitis media infections, were obtained from patients visiting hospitals and suffering from these infections. Traditional biochemical and morphological tests were performed to identify bacterial isolates. They were cultured on MacConkey, Blood, and Cetrimide agar (HiMedia). An optical microscope was used to examine morphological shape, size, and arrangement. Confirmation of the suspected isolates was achieved by utilizing the VITEK® 2 Compact system (bioMérieux, France).

Exotoxin A detection

An ELISA kit (BioTek Instruments, Inc.; serial no. 130131A) was used to investigate the production of Exotoxin A by the tested isolates.

Exotoxin A partial purification

The procedure performed in the current study was according to Whitaker and Bernhard (1972) (Whitaker & Bernhard, 1972). Different percentages (20, 30, 40, 50, 60, 70, 80, 90%) saturation of ammonium sulfate ((NH4)2SO4) were added to precipitate the toxin. Centrifugation was performed for 30 min at 10,000 rpm to separate the precipitate.

Ion exchange chromatography purification

The purification was carried out following the procedure described by Bradford (1976) (Bradford, 1976). A DEAE-cellulose column (2.5 x 13 cm) was used and washed several times with 0.01 M Tris-HCl buffer, pH 8, which acted as the equilibration buffer.

Determination of the molecular weight of exotoxin A

Gel filtration chromatography was applied to determine the molecular weight of the partially purified Exotoxin A produced by *P. aeruginosa* isolates. The toxin continuously flowed through a glass column (1.5 x 55 cm) filled with Sepharose 6B, eluted at a flow rate of 2 ml per fraction. Standard proteins used in this study included bovine serum albumin (BSA, 67 kDa), trypsin (23 kDa), and ovalbumin (43 kDa).

Estimation of Exotoxin A concentration

A Bovine Serum Albumin (BSA) standard curve was constructed by using various concentrations of BSA stock solution. This test was performed following the procedure described by Merghoub et al. (2009), where an ELISA kit was utilized (Merghoub et al., 2009).

Cell lines cultivation

Two distinct cell lines were employed in the current study: breast cancer (MCF-7) and normal human dermal fibroblast (HDFn). These cells were cultured in PRMI-1640 medium supplemented with 10% fetal bovine serum and 1% antibiotics (Nystatin, Benzyl Penicillin, and Streptomycin). The culture was placed in tissue culture flasks and incubated at 37°C with 5% CO_2 until a monolayer was formed. The trypan blue exclusion method was used to determine cell densities. Cells were counted until reaching a final concentration of 1×10^4 viable cells/ml.

Cytotoxicity Assay

Cell suspension was added to a 96-well microtiter plate at a density of 1×10^4 cells/ml. The final volume of complete culture media in each well was 200 µl. Plates were gently shaken, mixed, and incubated in a 5% CO_2 incubator at 37°C for 24 hours after being covered with sterile parafilm. After incubation periods of 24, 48, and 72 hours, plates were checked for formation of a confluent monolayer and contamination. The medium was then removed, and 200 µl of partially purified Exotoxin A at different concentrations (400, 200, 100, 50, 25 µg/ml) was added to the wells. Each concentration and the control group were tested in triplicate. Plates were incubated at 37°C with 5% CO_2 for 24 hours. Following exposure to Exotoxin A, 20 µl of MTT

solution was added to each well. Plates were then incubated for 4 hours at 37°C in 5% CO_2. Subsequently, the medium was carefully removed, and 200 µl of DMSO (solubilization solution) was added to each well and incubated for 5 minutes. Absorbance was measured at 570 nm using an ELISA reader. IC_{50} values were calculated based on optical density measurements according to the following equation: (%) Viability = (OD_{600} test / OD_{600} blank) × 100 (Chotirmall et al., 2012; Al-Rawi et al., 1986).

Results and Discussion

Current results showed that out of sixty-nine different clinical samples, including urinary tract infection (UTI), otitis media, wounds, and burn infections, fifty samples showed bacterial growth, while the remaining were negative for bacterial growth. Only thirty-one (62%) isolates belonged to *P. aeruginosa*, whereas the remaining isolates were thought to be related to other bacteria. Figure 1 illustrates the percentage of *P. aeruginosa* in different clinical samples.

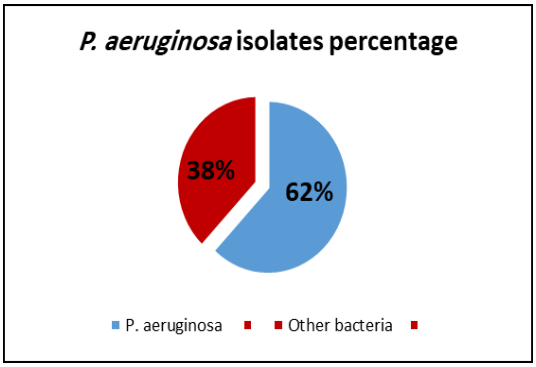

Fig 1. The percentage of *P. aeruginosa* in different clinical samples

These results differ from a recent study which reported a 19.3% prevalence of *P. aeruginosa* from wound, burn, otitis media, and UTI samples (Asamenew, 2023), and another study that revealed *P. aeruginosa* represented only 6.48% of isolates (Maharjan, 2022). Great concern has been raised regarding this bacterium due to its substantial repertoire of virulence factors, as well as its resistance to a wide range of antibiotics, which can result in a high mortality rate (Foulkes et al., 2022; Vidaillac & Chotirmall, 2021).

In a local study, it was found that 80% of *P. aeruginosa* isolates showed high ciprofloxacin resistance, whereas 100% were sensitive to imipenem (Yaseen and Ahmed, personal communication, 2023). Present results showed that burn infections had the highest bacterial colonization, followed by wounds, otitis media, and UTI infections, respectively. This finding agrees with Ali and Assafi (2024), who found that the highest rates of burn infections by *P. aeruginosa* reached 32.7% (Ali & Assafi, 2024).

Patients with burns are thought to be highly susceptible to nosocomial infections due to compromised immunity and the nature of their injuries (Risan et al., 2020). A wound is a break in the integrity of the skin's epithelium and can cause further disruptions to the skin's function, anatomy, and physiology (Che Soh et al., 2020). Figure 2 illustrates the prevalence of *P. aeruginosa* among a variety of clinical samples.

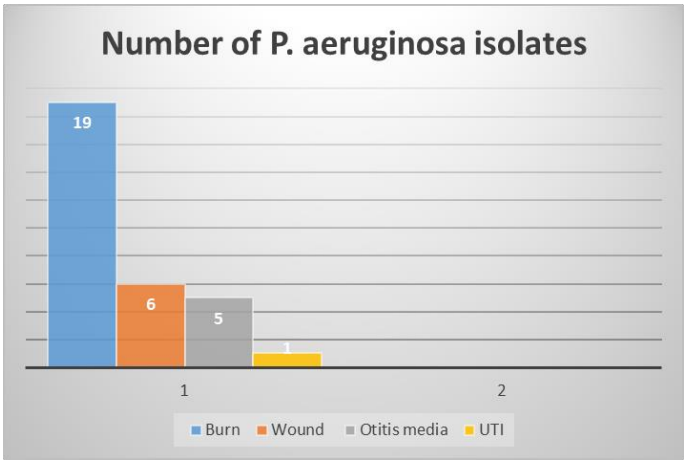

Fig 2. Prevalence of *P. aeruginosa* among various clinical samples.

Pathogenic bacteria such as *P. aeruginosa* can delay the proliferative phase of wound repair by secreting proteins that impair or delay healing (Prasad et al., 2020). *P. aeruginosa* appeared colorless when cultured on MacConkey agar due to its inability to ferment lactose. Additionally, Cetrimide agar was used to inhibit bacteria other than *P. aeruginosa* (Vermelho et al., 1996).

Various isolates were identified phenotypically based on colony morphology, pigment production, and biochemical tests such as nitrate reduction, sugar fermentation, oxidase test, catalase test, citrate utilization, motility, glucose oxidative-fermentative test, decarboxylase tests, and hemolysin production. These tests were performed according to standard protocols (CLSI, 2015; Koneman et al., 2006). The isolates showed β-hemolytic activity and were positive for oxidase, catalase, and citrate utilization tests, while negative for indole production and methyl red-Voges Proskauer tests.

Cultured colonies on nutrient agar at 42°C for 24 hours showed normal bacterial growth, considered a positive result. All bacterial isolates were protease positive, in agreement with Galdino et al. (2017), who reported that all 96 *P. aeruginosa* isolates were protease producers (Galdino et al., 2017). Pyocyanin production was tested on Cetrimide agar, which also serves as selective media for *P. aeruginosa* due to its cationic detergent action. This effect causes liberation of phosphorus and nitrogen that denatures the membrane proteins of bacteria other than *P. aeruginosa* (Vermelho, 1996).

Exotoxin A detection and concentration

Current results revealed that out of thirty-one *P. aeruginosa* isolates, only twenty-one were able to produce Exotoxin A. One isolate (P29) was selected based on its highest productivity of this toxin, reaching 29.24 ng/ml, and was associated with burn infections. This may be explained by the fact that isolates from burn infections may exhibit more virulence factors than those from other infections, hence the highest toxin production was observed in the burn infection isolate. Figure 3 represents Exotoxin A concentrations from different clinical sources.

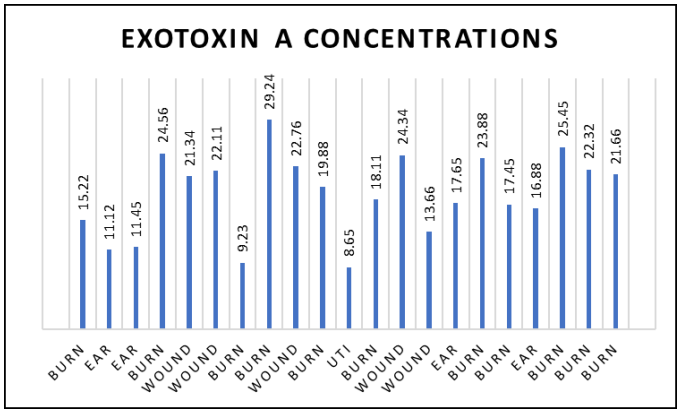

Fig 3. Exotoxin A concentrations detected in *Pseudomonas aeruginosa* isolates from different clinical sources.

Purification of Exotoxin A

Protein Precipitation by Ammonium Sulfate $(NH4)_2SO_4$)

To precipitate the crude toxin extract and remove water molecules, ammonium sulfate was used at different saturation percentages (20, 30, 40, 50, 60, 70, and 80%). Results showed that 80% saturation was optimal for precipitating Exotoxin A. Previous studies found that ammonium sulfate reduces protein solubility, resulting in precipitation due to salt-induced charge neutralization at the protein surface and disruption of the water layer surrounding the protein (Prinsloo et al., 2013; Pollack & Taylor, 1977).

Ion-Exchange chromatography for partial purification of Exotoxin A

Ion-exchange chromatography using DEAE-cellulose was employed for partial purification of Exotoxin A. As illustrated in Figure 4, Exotoxin A was detected during washing steps, and elution of fractions was observed. Although two peaks were identified, only one peak was observed for the elution of the P29 isolate, showing activity consistent with that measured by the ELISA kit. The partially purified protein concentration from P29 reached 29.24 ng/ml. These findings indicate that one peak after elution with gradient NaCl concentrations corresponds to purified Exotoxin A, with no other proteins separated.

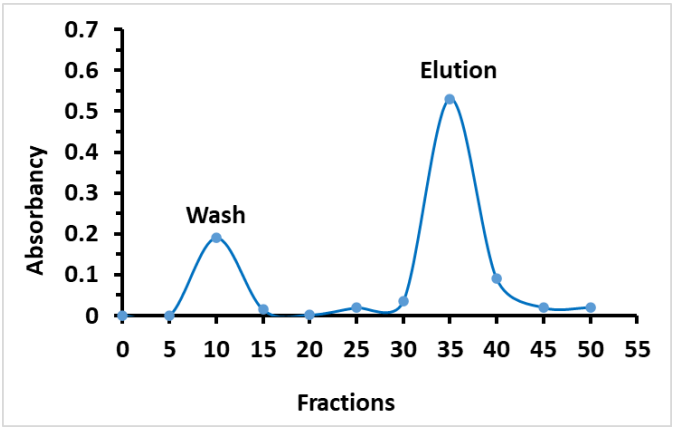

Fig 4. Ion-exchange chromatography profile showing the partial purification of Exotoxin A.

Exotoxin A molecular weight

The molecular weight of Exotoxin A was determined using Sepharose 6B gel filtration chromatography in the presence of trypsin, ovalbumin, and BSA as standard proteins. Each standard protein was separately eluted with Exotoxin A. The elution volume (Ve) was measured and reported as Ve/Vo. These results indicated that the molecular weight of Exotoxin A is approximately 65.33 kDa, as illustrated in Figure 5.

Exotoxin A cytotoxicity on MCF-7 cel line

The present results indicated that the viability of MCF-7 cells declined upon treatment. The highest concentration tested (400 µg/mL) caused the greatest inhibition of MCF-7 cells after 72 hours of incubation. This may be due to the incubation temperature influencing toxin A effector synthesis, with the greatest effector protein production occurring at 37°C (Saelinger et al., 1985). Another explanation is that gene induction

encoding the type III secretion system (TTSS) occurs at this temperature (Engel & Balachandran, 2009).

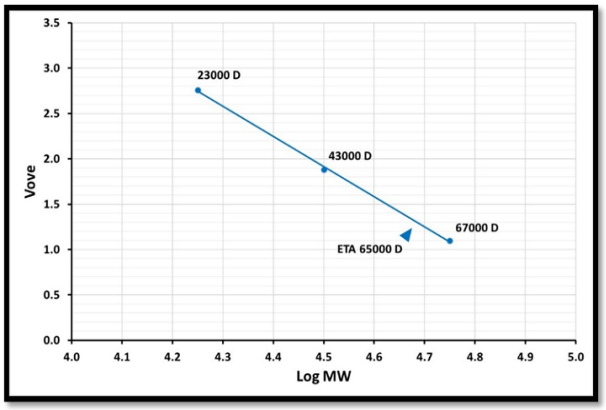

Fig 5. Determination of Exotoxin A molecular weight using gel filtration chromatography alongside standard proteins—trypsin (23 kDa), ovalbumin (43 kDa), and BSA (67 kDa).

This result aligns with a study reporting that purified Exotoxin A inhibits REF cell lines in a dose- and time-dependent manner, although inhibition of RD cell lines by Exotoxin A was time-independent (Saleh et al., 2013). The highest cytotoxic effect against MCF-7 cells was 68.3%, whereas the effect on normal human dermal fibroblast (HDFn) cells was low, with viability around 92%. This difference is likely due to apoptosis regulation mechanisms in cancer cells leading to cell death. Additionally, this damage may be linked to cytokine production, which acts as anti-inflammatory molecules and promotes phagocytosis (Prinsloo et al., 2013).

There is believed to be a strong correlation between the adherence and cytotoxicity of *P. aeruginosa* (Idziorok et al., 1990). Moreover, the contact-dependent type III secretion system (TTSS) depends on the number of bacteria adhering to eukaryotic cells, which correlates with the amount of translocated Exotoxin A effector (Beaumelle et al., 2001). Previous studies demonstrated that Exotoxin A biologically reduces proliferation of cancer cells (Nichenametla et al., 2006).

A local study found that isolates competent in Exotoxin A production showed inhibition ratios of 61.6% and 69.1% against PC3 and HeLa cancer cell lines, respectively, at the highest toxin concentration (Hassan, personal communication, 2021). However, the current results disagree with Kawakami et al. (2009), who reported that even low Exotoxin A concentrations (10 ng/ml) caused potent cytotoxicity in many cancer cell lines (Kawakami et al., 2009).

From these findings, it is clear that Exotoxin A has high cytotoxicity against MCF-7 cells, inhibiting cell proliferation and causing cell death.

Conflict of interest

The authors of this paper do not have any competing interests.

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