RESEARCH PAPER

Purification, Characterisation, and Cytotoxicity of Exotoxin A (*toxA*) from *Pseudomonas aeruginosa* Isolated from a Street Vegetable Sample.

Chinmayi A. [1]*, Usha M. S. [2]

[1*]Research scholar, Department of Microbiology and Botany, School of Sciences, JAIN (Deemed-to-be University), J. C. Road, Bengaluru -560027

[2] Professor, Department of Microbiology and Botany, School of Sciences, JAIN (Deemed-to-be University), J. C. Road, Bengaluru -560027

*Corresponding Author: chinnu10amar@gmail.com

ABSTRACT: *Pseudomonas aeruginosa* is an opportunistic pathogen capable of producing toxins that contribute to its virulence. This study details the purification and characterisation of toxins from *P. aeruginosa* isolated from a street vegetable sample. The purification process included ammonium sulphate precipitation, dialysis, ion exchange chromatography, and gel filtration chromatography. The protein concentration after the purification was 0.824 mg/m. Protein was further analysed using SDS-PAGE, revealing a band around 66 kDa. This band was excised and subjected to MALDI-TOF mass spectrometry for protein identification. MALDI-TOF-based protein identification identified the toxin as Exotoxin A with a score of 109, along with 12 % protein sequence coverage. The purified protein was then tested for cytotoxic effects. The protein exhibited moderate, dose-dependent anticancer activity against Caco-2 colon cancer cells, with an IC_{50} of approximately 970 µg/mL and caused minimal harm to normal HDF cells (IC_{50} >1000 µg/mL).

Keywords: *Pseudomonas aeruginosa*, exotoxin A, PCR, SDS PAGE, MALDI-TOF, cytotoxicity.

How to cite this article: Chinmayi A, Usha MS. Purification, characterisation, and cytotoxicity of exotoxin A (toxA) from Pseudomonas aeruginosa isolated from a street vegetable sample. Int J Drug Deliv Technol. 2026;16(73s): 1650-1663. DOI: 10.25258/ijddt.16.73s.176

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that can infect immunocompromised individuals. The virulence of the organism is due to its ability to colonise and form biofilms. Key virulence factors include pyocyanin, alkaline proteases, structural factors, hemolysins, and various toxins, including exotoxins, that promote tissue damage. *Pseudomonas aeruginosa* infections are associated with hospital-acquired infections. *Pseudomonas aeruginosa* can cause both acute and chronic infections, posing a major clinical challenge (Jawad & Rasheed, 2022)

The availability of water activity and a conducive pH, along with nutrients, helps opportunistic pathogens like *Pseudomonas aeruginosa* to grow and multiply on the fresh produce, thereby increasing the risk of contamination and leading to foodborne intoxication.

The pathogenicity of the organism mainly depends on the expression of multiple virulence genes, including exotoxin A (*toxA*), type III secretion systems (*exoS, exoT, exoU,* and *exoY*), and elastases (*lasA* and *lasB*). These factors contribute to the main cell injury by disrupting cellular structure, inhibiting protein synthesis, and promoting the degradation of extracellular matrix components. Because virulence genes are distributed variably among isolates, molecular detection by PCR plays a major role in identifying virulence strains and assessing their virulence potential (Veetilvalappil *et al.,* 2022; Qin *et al.,* 2022).

Purification of bacterial proteins is essential for determining their biological properties. Extracellular bacterial toxins have attracted significant interest due to their biological activity and potential therapeutic uses. The toxins and their activity directly interfere with fundamental cellular processes such as signal transduction, membrane integrity, protein synthesis, and cell death. For these reasons, these toxins are considered candidates for the development of anticancer drugs that induce apoptosis while having minimal effects on normal cells, making it important to study bacterial toxins and their protein-based therapeutics (Qin *et al.,* 2022).

This study aimed to examine the virulence traits of a *Pseudomonas aeruginosa* isolate by PCR detecting specific virulence genes. The extracellular toxin was purified by ammonium sulphate precipitation, dialysis, ion-exchange chromatography, and gel-filtration chromatography. The purified protein was characterised by SDS-PAGE and identified via MALDI-TOF mass spectrometry. The toxin's cytotoxicity was assessed against normal human dermal fibroblasts (HDFs). At the same time, its anticancer potential was evaluated against human colorectal adenocarcinoma (Caco-2) cells to explore its suitability as a selective bioactive protein for therapeutic use.

MATERIALS AND METHODS

Pseudomonas aeruginosa culture: The *Pseudomonas aeruginosa* culture (S07VO-1) isolated from a street

*Author for Correspondence: chinnu10amar@gmail.com

onion sample, was inoculated onto King's B Media and maintained for further studies (Fig. 1).

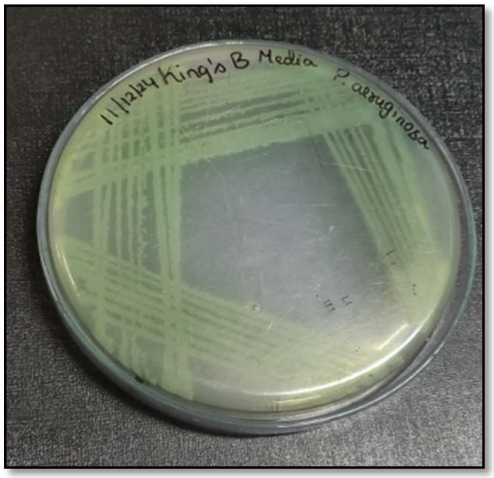

Fig. 1: Growth of *Pseudomonas aeruginosa* (S07VO-1) on King's B Media

Genomic DNA and PCR analysis: Genomic DNA was extracted**,** and PCR analysis was performed to detect the virulence gene in *Pseudomonas aeruginosa* (S07VO-1). The isolate was tested for the specific toxin-producing toxA gene relevant to its pathogenic profile using organism-specific primer sets. The primers and the gene associated with the primers are *toxA* Forward primer: 5'- GGTAACCAGCTCAGCCACAT-3' and *toxA* Reverse primer: 5'-TGATGTCCAGGTCATGCTTC-3'. The PCR was performed in a total volume of 25 µl, and the components are listed in Table 1 (Bogiel *et al*., 2021).

Table 1: Components of PCR amplification

Components	**Quantity(µL)**
Template	2 µL (~ 50 ng of DNA)
Primer (F/R)	2 µL (~ 5 pmol)
Master mix	13 µL
Water	6 µL

Amplification was conducted using a thermal cycler with an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 45 seconds. A final extension was performed at 72°C for 5 minutes. Amplified products were analysed by agarose gel electrophoresis, and detection of the expected amplicon size confirmed the presence of the targeted toxigenic gene. The toxA gene was amplified using conventional PCR with an optimised annealing temperature (Bogiel *et al*., 2021).

Toxin purification: The toxin from the (*P. aeruginosa*, S07VO-1) isolate was partially purified using ammonium sulphate precipitation, dialysis, ion-exchange chromatography and gel filtration.

Inoculum preparation: A loopful of bacterial culture (*P. aeruginosa*, S07VO-1) was inoculated into freshly autoclaved 50 mL of King's B medium and incubated for 24 hours at 37 °C.

Production of exotoxin: 1000 mL of King's B medium was inoculated with 5% of the inoculum and incubated for 24 hours at 37 °C. Extraction of exotoxin: The production media was centrifuged at 5000 rpm for 20 minutes. The cell-free supernatant was then collected, and 12.5 mL of 1 M zinc acetate was added. The mixture was incubated at 4 °C for 1 hour to precipitate the toxin, followed by a second centrifugation at 5000 rpm for 20 minutes. The pellet was dissolved in 0.3 M sodium citrate. The toxin in sodium citrate was then dialysed against pH 8.0 Tris-HCl buffer at 4ºC for 24 hours. After dialysis, the dialysate was centrifuged at 5000 rpm for 20 minutes to separate insoluble materials.

Ammonium sulphate precipitation: The 900 mL supernatant from the extraction was used for salt precipitation. Ammonium sulphate was added gradually, in small amounts, to reach 40% saturation- specifically, 226.8 g while maintaining ice-cold conditions and stirring continuously at 1000 rpm with a magnetic stirrer. The pellet obtained was dissolved in 20 mL of 1X phosphate-buffered saline (PBS) containing 10 mM phosphate (Duong-Ly *et al*., 2014) (Fig. 2).

Fig. 2: Ammonium sulphate precipitation of the sample

Dialysis: Dialysis was performed for the protein (P1) sample obtained after 40% saturation. The dialysis process was initiated by activating the dialysis membrane. 100 mL of distilled water was boiled, a dialysis membrane (flat width: 37.70 mm, diameter: 25.4 mm, capacity approx.: 5.07 mL/cm) of 8 cm was added, and the mixture was boiled for 10 minutes. 2 gm of sodium bicarbonate was added pinch by pinch, allowing it to boil for 10 minutes. The membrane was transferred to fresh boiling water and boiled for 10 minutes. The activated dialysis bag was filled (9 mL, 40% saturation suspension) with the re-suspended solution from the previous ammonium sulphate precipitation step and sealed on both sides, ensuring it was free from any air bubbles. The bag was kept in the container filled with deionised water and incubated overnight at 4°C. The unit was placed on the magnetic stirrer and stirred continuously. The deionised water was changed every half an hour. The dialysis lasted 2 hours. Later, the dialysis bag was punctured, and the sample was procured for further purification (Fig. 3).

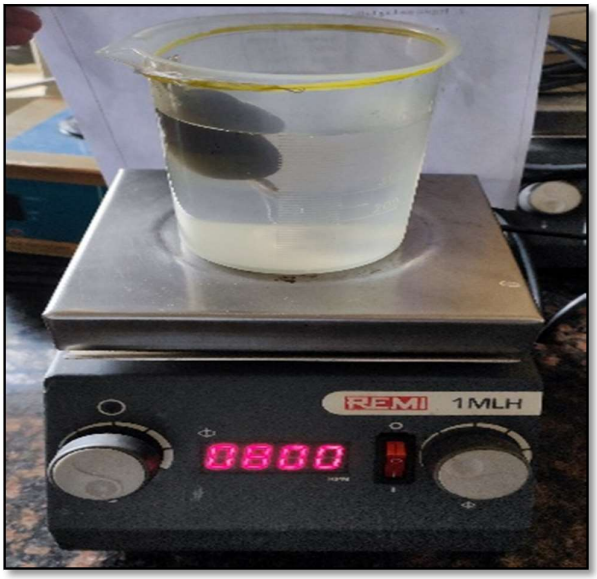

Fig. 3: Dialysis of the sample

Ion exchange chromatography: The dialysed sample was processed for anion-exchange chromatography. The activated anion-exchange matrix, DEAE cellulose (1 g in 10 mL of double-autoclaved distilled water), was poured into the column (Sintered Silica G0) with a diameter of 10 mm and a length of 25 cm. Six elution buffers of different concentrations, such as 25 mM, 50 mM, 75 mM, 100 mM, 125 mM, and 150 mM, were prepared using a 1 M NaCl stock solution by adding 200 µL, 400 µL, 600 µL, 800 µL, 1000 µL, and 1200 µL, respectively. A total volume of 200µL of 1 M Tris-HCl was added to all the elution buffers, and the final volume was made up to 8 mL with autoclaved water (Saleh *et al.,* 2012) (Fig. 4)

Fig. 4: DEAE-Cellulose Anion Exchange Chromatography of the sample

Gel filtration: Protein 1 (P1) was purified using gel filtration. P1 was purified using Sephadex G-75, chosen for high-molecular-weight fractionation, with a 13 mm radius × 30 cm length (≈159.2mL bed volume). The loading sample volume was 8 mL, approximately 5% of the bed volume. The elution buffer used was 50 mL of 100 mM Phosphate Buffer (pH 7.0). The flow rate was 1 mL per minute. The A_{280} method was used to quantify protein (Fig. 5) (Pharmacia Fine Chemicals, 1970).

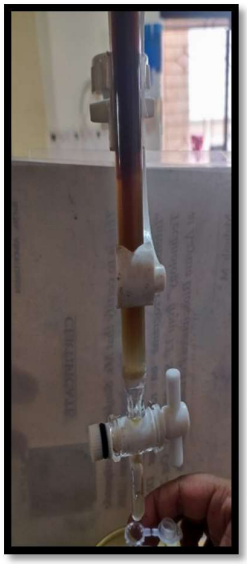

Fig. 5: Gel filtration column for purification of toxin (P1)

SDS-PAGE: The protein samples were analysed via SDS-PAGE using an 18% polyacrylamide separating gel and a discontinuous buffer system as originally described by Laemmli (1970).

Characterisation of toxin using MALDI-TOF analysis: Purified protein was subjected to SDS-PAGE. The protein band corresponding to the target protein was excised and submitted to the institutional proteomics facility for in-gel tryptic digestion and MALDI-TOF peptide mass fingerprinting. The purified protein was analysed using a rapifleX MALDI-TOF mass spectrometer (Bruker Daltonics, Germany). The peptide solution was combined with an equal volume of 2,5-dihydroxybenzoic acid (DHB) matrix and then spotted onto the MALDI target plate. The matrix helped in the desorption and ionisation of the peptides when exposed to the laser. The mass spectrum was recorded in positive ion reflectron mode using FlexControl software, which provided accurate peptide mass measurements. The peptide mass fingerprint obtained was searched against the SwissProt database using the Mascot search engine with trypsin selected as the digestion enzyme. The protein was identified by evaluating the Mascot score, matching peptide masses, and considering the statistical significance of the search results.

Cytotoxicity study: A cytotoxicity study was carried out by MTT assay using a human dermal fibroblast cell line and Human colorectal adenocarcinoma cell lines.

Maintenance of Cell Line: The Caco-2 cell line (Human colorectal adenocarcinoma) was purchased from NCCS, Pune, India. The cells were maintained in MEM with NEAA mixture supplemented with 10% FBS along with

1% antibiotic-antimycotic solution in an atmosphere of 5% CO_2, 18-20% O_2 at 37 °C in the CO_2 incubator and sub-cultured every 2 days. The passage number of Caco-2 was 35 and was used for the present study.

The HDF (Human dermal fibroblast cell line) was purchased from Himedia Labs, Mumbai, India. The cells were maintained in DMEM with high glucose supplemented with 10% FBS along with 1% antibiotic-antimycotic solution in an atmosphere of 5% CO_2, 18-20% O_2 at 37 °C in a CO_2 incubator and sub-cultured every 2 days. HDF passage 8 was used for the present study.

After reaching 80% density, cells were detached using 0.025% trypsin and 0.01% EDTA (in D-PBS). Cell viability and count were performed using a hemocytometer. The appropriate cell density was seeded in T25 flasks and cultured until further use for cell-based assays.

Cell viability and proliferation studies - MTT assay: The cytotoxic effect of the test compound (P1) was analysed in Caco-2 and HDF cells by performing the MTT assay. MTT is a tetrazolium salt which is converted into insoluble, purple-coloured formazan crystals by the action of lactate dehydrogenase enzyme released by mitochondria. Briefly, cells in 200 µl of suitable media, at a density of 15,000, were plated in a 96-well plate and incubated at 37°C overnight. After cell attachment to the cell culture plate, the spent medium was replaced with complete medium containing the test compound at final concentrations of 15.62, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL. Cisplatin at 10 µg/mL was used as a reference standard for the study. After drug addition, cells were incubated for 24 h at 37°C with a 5% CO_2 atmosphere. After incubation, 100 µl of MTT (0.5 mg/mL) was added and incubated for 3 h at 37°C. DMSO (100µL) was used to dissolve the formazan crystals, and the purple colour was measured at 570 nm using a microplate reader (ELX-800, BioTek, USA). The viability of cells treated with DMEM alone was considered as 100%. Percentage cell viability was calculated using the formula below:

Percentage cell viability = [OD of treated cells/OD of Untreated cells] x 100

The IC_{50} value was determined using a linear regression equation, i.e., Y = Mx + C. Here, Y = 50, and M and C values were derived from the viability graph.

RESULTS

PCR Analysis: On PCR amplification, *Pseudomonas aeruginosa* isolate S07VO-1 showed positive results for the *toxA* virulence gene. A band that appeared between 300 bp and 400 bp in lane 6 could be the amplified *toxA* product (Fig. 6).

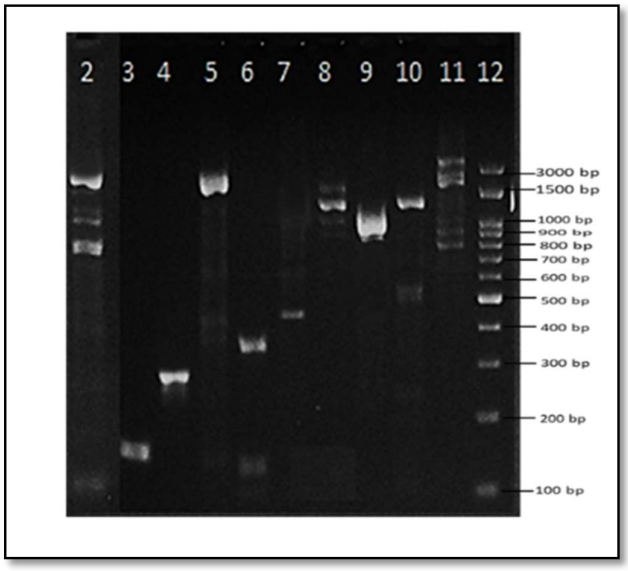

Fig. 6: Agarose gel electrophoresis of PCR products from the isolate S07VO-1

Note: Lane 6: PCR amplification product of the *toxA* gene, Lane 12: Marker DNA

Toxin purification: The purification profile of Exotoxin A obtained from *Pseudomonas aeruginosa* is presented in Table 2. The culture supernatant (900 ml) served as the crude extract and contained a total protein concentration of 5.457 mg/mL, corresponding to 4911.30 mg of total protein, which was considered 100% recovery.

Following ammonium sulphate precipitation, the protein concentration increased to 7.457 mg/mL, while the total protein decreased to 149.14 mg, resulting in a recovery of 3.04%. This reduction in total protein indicates the removal of a substantial proportion of unwanted proteins during the initial precipitation step.

After dialysis, the protein concentration decreased to 5.057 mg/mL, with a total protein content of 45.51 mg and a recovery of 0.93%. The decrease in total protein after dialysis was attributed to the removal of residual

ammonium sulphate and other low-molecular-weight impurities, together with minor protein losses during dialysis.

Purification by DEAE-cellulose ion-exchange chromatography further reduced the total protein to 19.29 mg, with a protein concentration of 2.411 mg/mL and a recovery of 0.39%. Fraction 1 was selected for further purification based on its protein profile.

The selected ion-exchange fraction was subsequently subjected to Sephadex G-75 gel filtration chromatography. Three consecutive fractions (GF1–GF3) corresponding to the protein peak were pooled, yielding a final volume of 3 mL with an average protein concentration of 0.824 mg/mL. The final pooled Sephadex G-75 fraction contained 2.47 mg of total protein, corresponding to a final total protein recovery of 0.05% after sequential purification (Table 2) (Fig. 7).

Overall, the purification process resulted in a progressive decrease in total protein and recovery percentage, reflecting the sequential removal of unwanted proteins during ammonium sulphate precipitation, dialysis, ion-exchange chromatography, and gel-filtration chromatography.

Table 2: Purification profile of Exotoxin A from *Pseudomonas aeruginosa*

Purification step	Protein concentration (mg/mL)
Crude extract	5.457
Salt precipitate	7.457
Dialyzed sample	5.057
DEAE-cellulose Fraction 1	2.411
Pooled Sephadex G-75 fractions	0.824

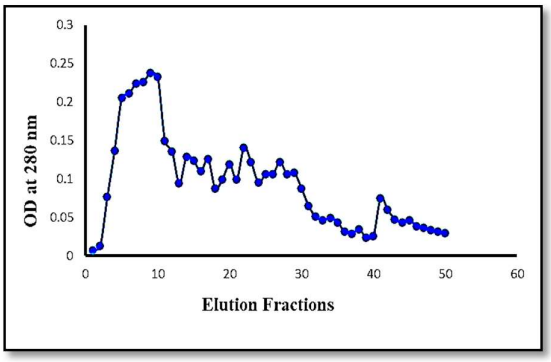

Fig 7: Elution profile of *Pseudomonas aeruginosa* toxin (P1) obtained via gel filtration chromatography

SDS-PAGE: The SDS-PAGE results showed the step-by-step purification of Exotoxin A, resulting in a purified protein with an estimated molecular weight of ~66 kDa, ready for further analysis (Fig. 8).

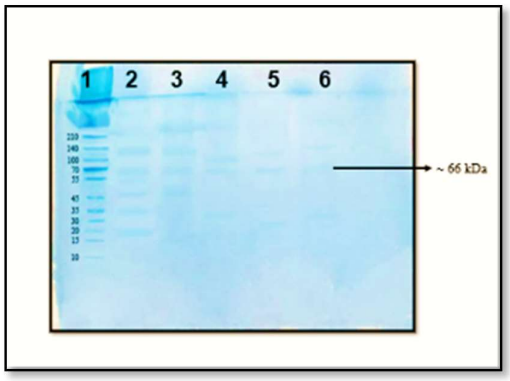

Fig 8: SDS-PAGE analysis of *Pseudomonas aeruginosa* toxin (P1) at various purification stages

Note: Lane 1: Protein marker, Lane 2: Crude, Lane 3: Ammonium sulphate precipitation, Lane 4: Dialysis, Lane 5: Ion exchange chromatography, Lane 6: Gel filtration (~ 66 kDa)

Characterisation of toxin using MALDI-TOF: The MALDI-TOF peptide mass fingerprinting found Exotoxin A from *Pseudomonas aeruginosa* with a Mascot protein score of 109, exceeding the significance

threshold (70). The protein was identified with an E-value of 7.2×10^{-6} and 12% sequence coverage. Four representative peptide ions at m/z 769.4566, 855.4359, 950.4366, and 2574.3139 matched the theoretical tryptic peptides VLAGNPAK, DATTFVR, WSEWASGK, and GYEVGYHGTFLEAAQSIVFGGVR, respectively. These peptide matches, together with the overall Mascot score, support the identification of Exotoxin A (Fig. 9-12).

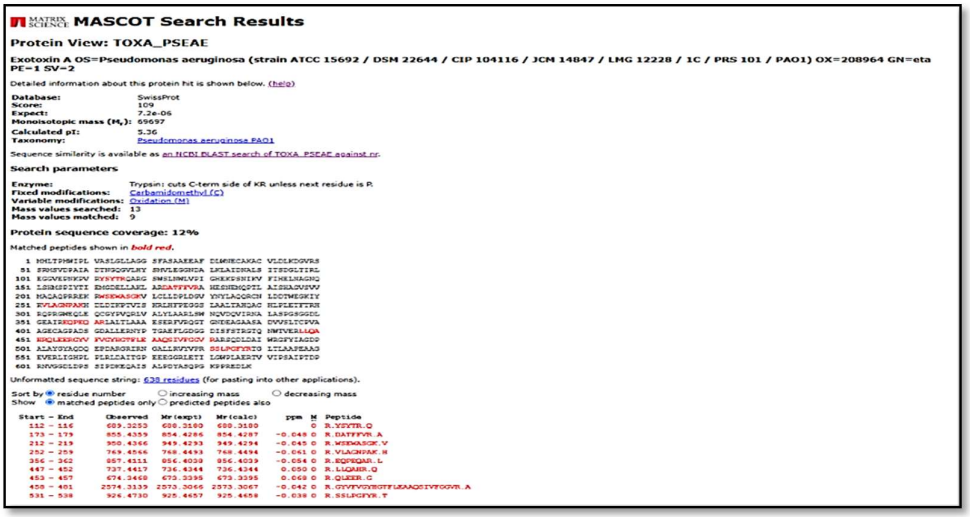

Fig 9: MASCOT search results with molecular weight and protein sequence coverage for *toxA* toxin from the *Pseudomonas aeruginosa* isolate S07VO-1

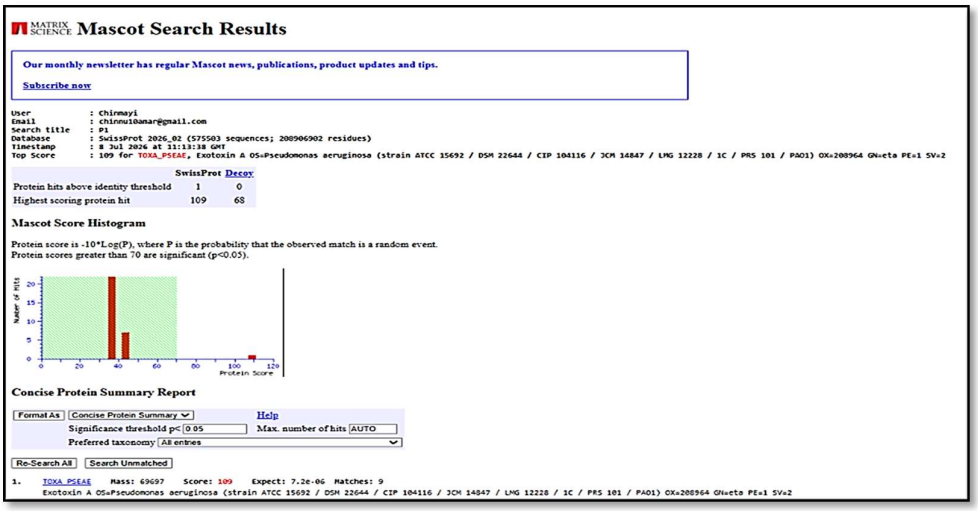

Fig 10: MASCOT search results with histogram and strain identification for the *Pseudomonas aeruginosa* isolate S07VO-1 toxin *toxA*

Note: OS= Organism name, GN= Gene name, PE= Protein Existence, SV= Sequence Version

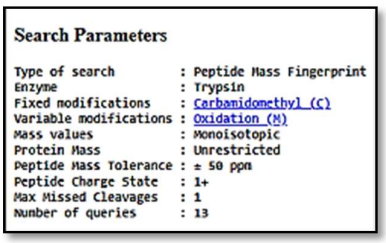

Fig 11: Mascot database search parameters used for protein identification

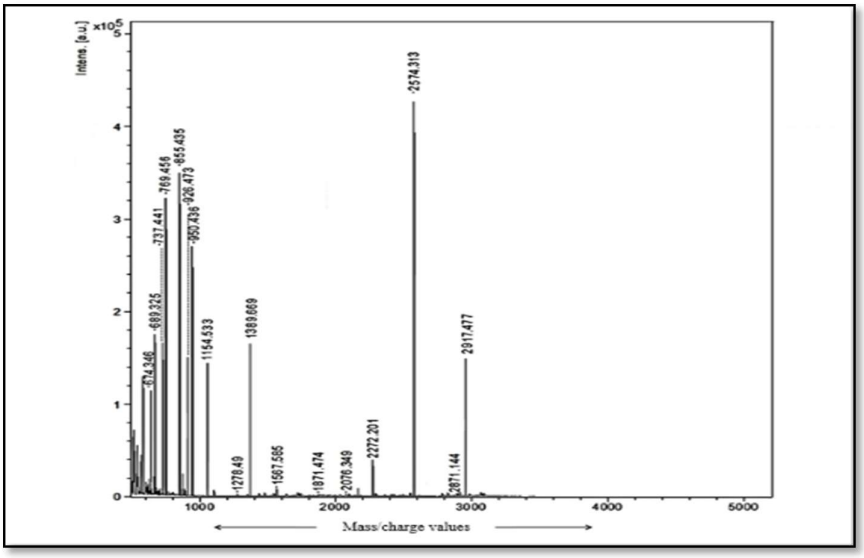

Fig. 12: MALDI-TOF spectrum used to identify the *Pseudomonas aeruginosa* protein (P1) associated with *toxA* toxin

Cytotoxicity study: The cytotoxic effect of compound P1 was evaluated in Caco-2 (human colon cancer) and HDF (normal human dermal fibroblast) cells using the MTT cell viability assay after 24 hours of treatment. P1 exhibited significantly lower cytotoxicity in normal HDF cells. Cell viability remained above 90% across concentrations from 15.62 µg/mL to 500 µg/mL, with some doses showing 100% viability, indicating possible stimulation of cellular metabolic activity or proliferation at moderate levels. At 1000 µg/mL, viability was still 54.70%, notably higher than in Caco-2 cells at the same dose. The IC_{50} for HDF cells exceeded 1000 µg/mL, suggesting lower toxicity to normal cells. Cytotoxicity assays with cisplatin (10 µg/mL) showed a viability decrease to 40.80%, underscoring its non-selective toxicity compared to P1.

Caco-2 cells showed a concentration-dependent decline in viability. At lower concentrations (15.62–125 µg/mL), viability remained high, ranging from 99.31% to 94.59%, suggesting minimal cytotoxicity. As the concentration increased to 250 µg/mL and 500 µg/mL, viability decreased to 91.17% and 82.37%, respectively. A significant drop to 47.93% was observed at 1000 µg/mall Linear interpolation estimated the IC_{50} of P1 in Caco-2 cells at about 970 µg/mL, meaning a relatively high dose is needed to inhibit 50% of the cells. The positive control, cisplatin (10 µg/mL), lowered viability to 66.90%, confirming the assay's effectiveness.

Overall, the data indicate that P1 shows selective cytotoxic activity, inhibiting Caco-2 colon cancer cells more effectively than normal HDF cells, especially at higher concentrations. The different IC_{50} values highlight the compound's possible selectivity (Table 3 and 4) (Fig. 13-16).

Table 3: Percentage cell viability of HDF cells treated with various concentrations of P1 along with the IC_{50} value determined after 24 hours of treatment.

MTT Assay-Summary-P1 vs HDF		
Drug conc. (µg/mL)	**% cell viability**	**IC_{50} conc. (µg/mL)**
Untreated	100	**>1000 µg/mL**
Cisplatin-10 µg	40.80	
P1-15.62 µg	92.62	
P1-31.25 µg	96.63	
P1-62.5 µg	100.54	
P1-125 µg	103.24	
P1-250 µg	104.41	
P1-500 µg	113.32	
P1-1000 µg	54.70	

Table 4: Percentage cell viability of Caco-2 cells treated with various concentrations of P1 along with the IC_{50} value determined after 24 hours of treatment

MTT Assay-Summary-P1 vs Caco-2		
Drug conc. (µg/mL)	**% cell viability**	**IC_{50} conc.(µg/mL)**
Untreated	100	**970 µg/mL**
Cisplatin-10 µg	66.90	
P1-15.62 µg	99.31	
P1-31.25 µg	98.66	
P1-62.5 µg	96.16	
P1-125 µg	94.59	
P1-250 µg	91.17	
P1-500 µg	82.37	
P1-1000 µg	47.93	

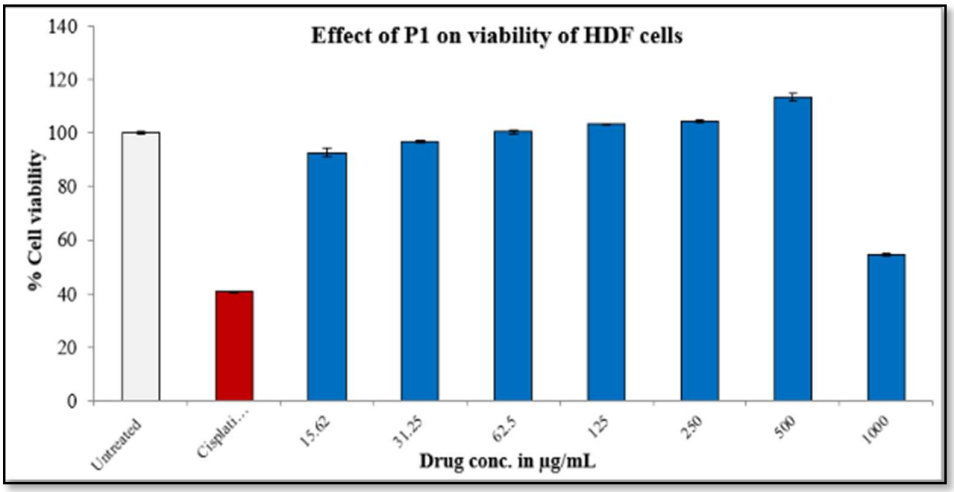

Fig 13: Overlaid bar graph showing the percentage of cell viability in HDF cells treated with different concentrations of P1 after 24 hours of incubation.

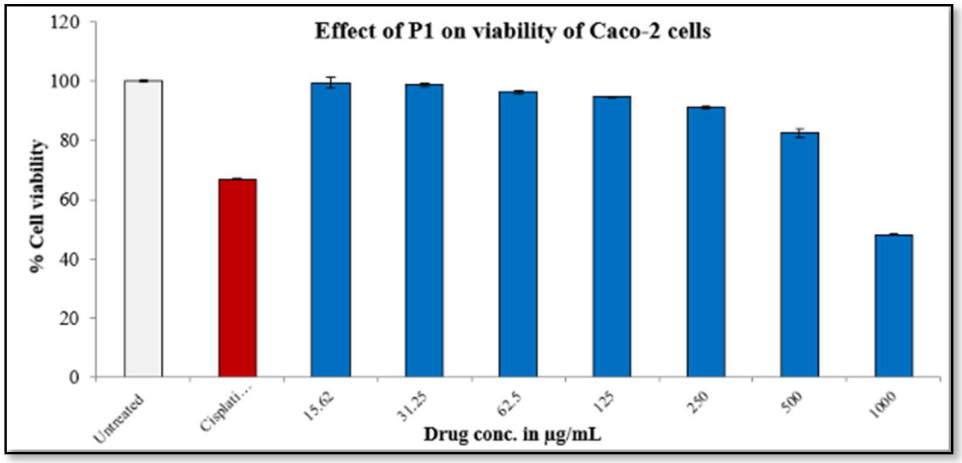

Fig 14: Overlaid bar graph showing the percentage of cell viability in Caco-2 cells treated with different concentrations of P1 after 24 hours of incubation.

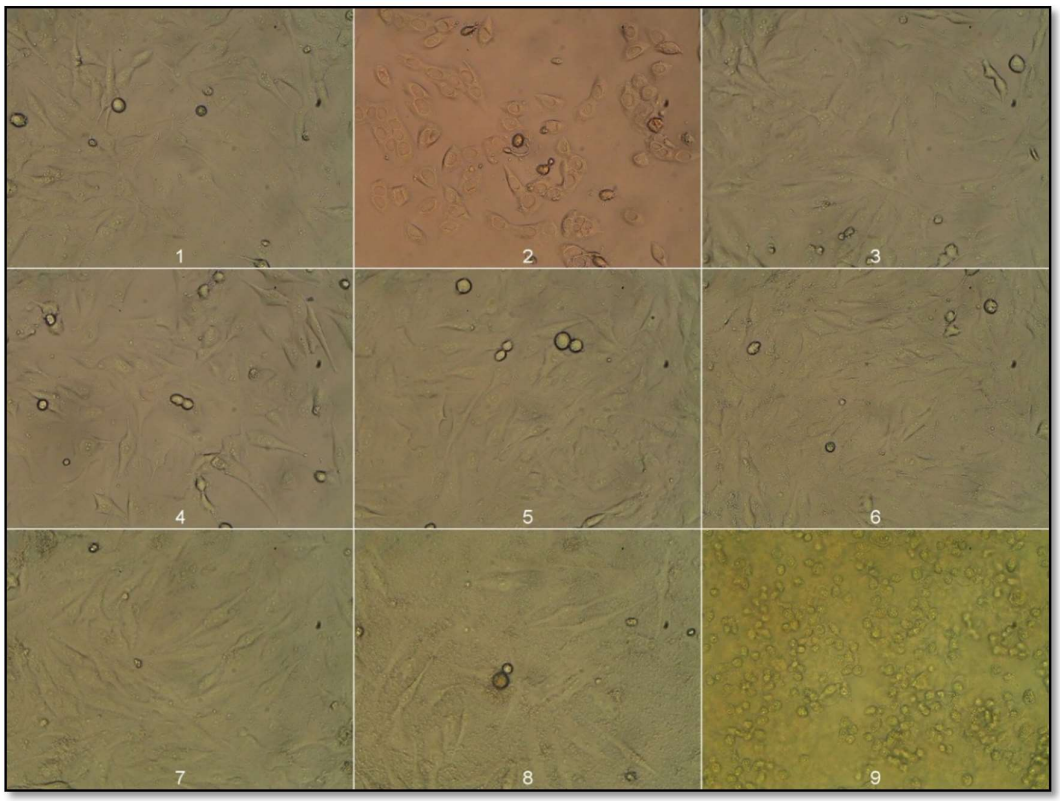

Fig 15: Overlaid montage images showing the morphology of HDF cells treated with varying concentrations of P1 after 24 hours of incubation. All images were captured at 20× magnification using an inverted biological microscope and recorded with a digital camera equipped with MICAM software.

Legend: 1-Untreated, 2-Cisplatin-10 ug/mL, 3-9: P1 at varying concentrations (15.62 µg/mL to 1000 µg/mL)

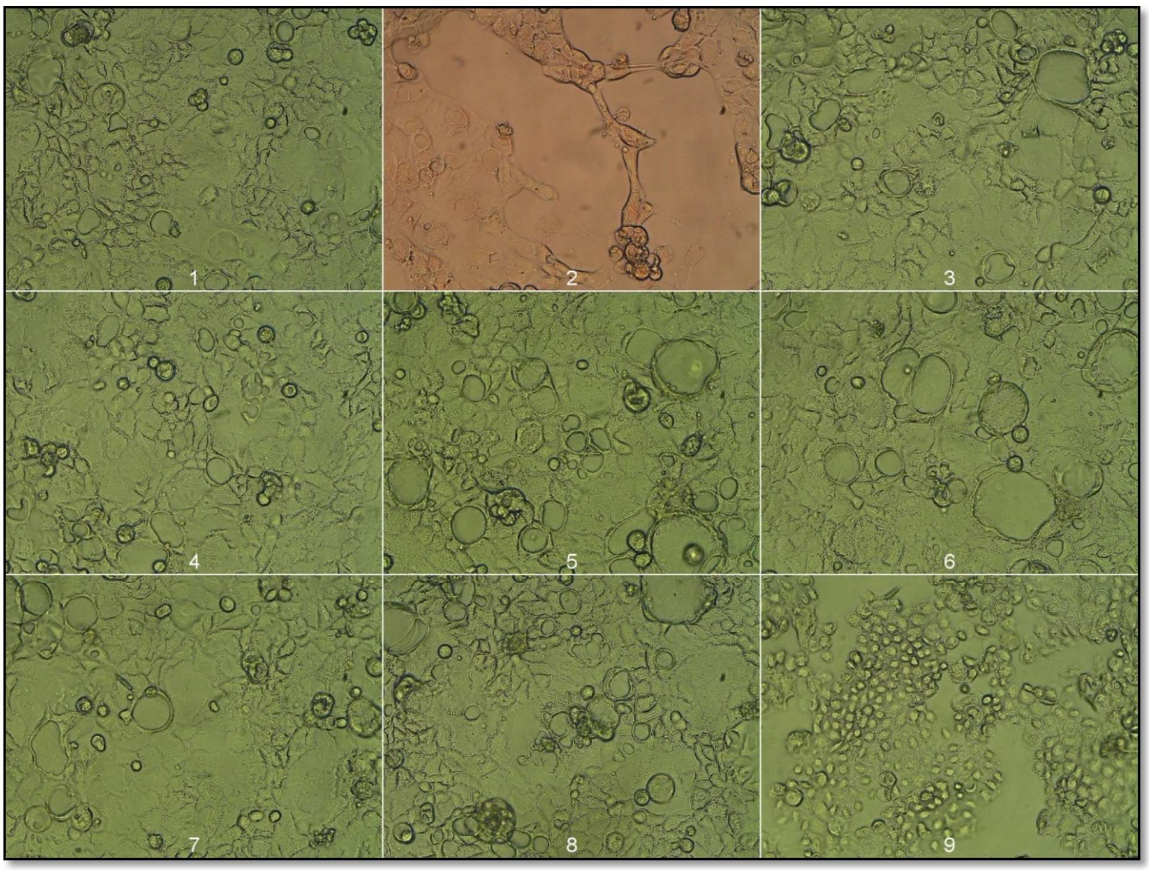

Fig 16: Overlaid montage images showing the morphology of Caco2 cells treated with varying concentrations of P1 after 24 hours of incubation. All images were captured at 20× magnification using an inverted biological microscope and recorded with a digital camera equipped with MICAM software.
Legend: 1-Untreated, 2-Cisplatin-10ug/mL, 3-9: P1 at varying concentrations (15.62 µg/mL to 1000 µg/mL)

DISCUSSION

The present study investigated the virulence genes of *Pseudomonas aeruginosa*. The PCR analysis mainly focused on the *toxA* gene and tested positive for the same.

Ema *et al.* (2022) performed identification using API 20E (Biomerieux, France), which identifies Enterobacteriaceae and other Gram-negative rods, including *E. coli.* This study mainly focused on the stxs-1 and srtx-2 genes. Of the 21 samples that contained *E. coli*, none tested positive for both the stx-1 and stx-2 genes.

The study conducted by Al-Tememe and Abbas (2022) assessed the molecular confirmation of the *aroE* gene in 42 bacterial isolates, yielding positive results with a gene size of ≈ 495 bp.

The study conducted by Thabit *et al.* (2025) reported a high prevalence of type III secretion system exotoxin genes in *Pseudomonas aeruginosa* isolates. The *exoS* gene was the most frequently detected, reported in 70 isolates, followed by *exoY* in 68 and *exoU* in 28. Of the 66 isolates resistant to carbapenems, 94% carried the *exoS* gene, followed by 91% carrying the *exoY* gene, 86% carrying the *exoT* gene, and 39% carrying the *exoU* gene, the least detected.

Almami (2025) examined 6 *Pseudomonas aeruginosa* isolates for the Exotoxin A (*toxA*) gene using PCR. All 6 isolates carried the toxA gene and were confirmed by the expected amplicon size of 367 bp. The test was also conducted on the same isolates after mutation induction; all the isolates confirmed the presence of a 367 bp amplicon size post-PCR. Due to the presence of an extra band, suggesting a genetic alteration in mutant isolate number 4.

The current study assessed the purification profile of Exotoxin A through a series of steps, including ammonium sulphate precipitation, dialysis, DEAE-cellulose ion-exchange chromatography, and Sephadex G-75 gel filtration. Each step gradually reduced the total protein content while enriching the target protein. Starting with 4911.30 mg in the crude extract, the protein decreased to 149.14 mg after ammonium sulphate precipitation, 45.51 mg after dialysis, 19.29 mg following DEAE-cellulose chromatography, and finally 2.47 mg in the pooled Sephadex G-75 fractions. The total protein recovery decreased from 100% in the crude extract to 0.05% in the final pooled fraction, indicating

the removal of contaminants during purification. Despite the low overall recovery, the final pooled fraction was sufficiently purified by SDS-PAGE, exhibiting a band around 66 kDa, and subsequent MALDI TOF-based protein identification identified the protein as Exotoxin A with a score of 109, along with 12% protein sequence coverage.

The purification of Exotoxin A was carried out by Almami (2025) using 70% ammonium sulphate precipitation, dialysis, and gel filtration chromatography with Sephadex G-150. The protein was confirmed by SDS-PAGE, with an approximate molecular weight of 66 kDa.

Abdelaziz *et al*. (2022) studied pyocyanin production by *Pseudomonas aeruginosa,* culturing the isolate in peptone water with 0.3% cetrimide at pH 8, achieving a yield of 53 µg/ml. The pyocyanin was purified by silica gel column chromatography and confirmed as pure by HPLC, which showed the same retention time (5.033).

Conice and Usha (2017) purified a toxin from an *Escherichia coli* isolate through sequential cation- and anion-exchange chromatography. Sepharose 6B was utilized as the matrix for anion exchange, whereas carboxymethyl cellulose was employed for cation exchange. The maximum protein concentrations in the eluted fractions were 3.55 mg/mL for the anion-exchange fraction and 2.76 mg/mL for the cation-exchange fraction. SDS-PAGE analysis identified two distinct bands with approximate molecular weights of 33 kDa and 7 kDa.

Marey *et al.* (2024) investigated the extraction and characterisation of pyocyanin from clinical isolates of *Pseudomonas aeruginosa*. Out of 30 isolates, 13 contained both the phzM and phzS genes and produced pyocyanin at concentrations ranging from 4.95 to 9.90 µg/ml. The extracted pyocyanin was identified using UV-Vis spectrophotometry, FTIR, GC-MS, and LC-MS, confirming its chemical structure, with a molecular formula of $C_{11}H_8N_2O$ and an estimated molecular weight of approximately 210 Da.

Hoyt *et al.* (2024) validated an Endopep-MS MALDI-TOF assay for detecting and characterising botulinum neurotoxins (BoNT/A, B, E, and F). The assay identified serotype-specific peptide cleavage products with detection limits comparable to or surpassing those of the traditional mouse bioassay. It demonstrated 100% specificity, accurately identified 19 distinct BoNT subtypes, achieved 99.9% reproducibility, and attained a 99.4% accuracy rate in blind multicenter evaluations. These results confirm the effectiveness of MALDI-TOF for bacterial toxin detection and analysis.

In the present study, P1 exhibited moderate, dose-dependent anticancer activity against Caco-2 colon cancer cells, with an IC_{50} of approximately 970 µg/ml and caused minimal harm to normal HDF cells (IC_{50} >1000 µg/ml). These findings suggest that P1 selectively targets cancer cells while having low toxicity to healthy cells. Nevertheless, since the effective dose is relatively high, further research- including mechanistic studies, apoptosis assays, and dose optimisation is necessary to more thoroughly assess its therapeutic potential.

The purified exotoxin A study of a mutant variant by Almami (2025) exhibited greater cytotoxicity against breast cancer cells, with IC_{50} values decreasing from 13.1 to 4.9 µg/mL for the wild-type isolate and from 12.0 to 3.6 µg/mL for the mutant isolate, indicating that purification enhanced its biological activity.

Abdelaziz *et al.* (2022) examined the cytotoxic effects of pyocyanin using the MTT assay. They found that purified pyocyanin specifically inhibited MCF-7 breast cancer cells, with an IC_{50} of 15 µg/ml, and showed no toxicity to normal peripheral blood mononuclear cells (PBMCs) at doses below 45 µg/ml. The study also revealed that pyocyanin induced significant apoptosis and necrosis in MCF-7 cells, as confirmed by confocal microscopy, flow cytometry, morphological analysis, and increased caspase-3 levels. This indicates that its anticancer effect likely involves caspase-3-dependent apoptosis.

In their study, Marey *et al*. (2024) performed cytotoxicity tests that showed pyocyanin's anticancer effects depend on dose and exposure time. After 48 hours, the IC_{50} values were 130 µg/mL for A549 lung cancer cells, 105 µg/mL for MDA-MB-231 breast cancer cells, and 187.9 µg/mL for Caco-2 colorectal cancer cells. Importantly, pyocyanin was less harmful to normal fibroblasts, with an IC_{50} of 194.1 µg/mL, indicating moderate selectivity for cancer cells.

Conclusions: This study's findings are important for understanding how *P. aeruginosa* causes disease and provide a strong basis for future research. Such studies might examine toxin-host interactions, develop anti-virulence therapies, design vaccines, identify diagnostic biomarkers, and create toxin-based treatments. Further research involving quantitative proteomics, structural analysis, recombinant expression, and in vivo testing will enhance understanding of Exotoxin A and help its use in medicine and clinical applications.

References

1. Abdelaziz, A. A., Abo Kamer, A. M., Al-Monofy, K. B., & Al-Madboly, L. A. (2022). A purified and lyophilized *Pseudomonas aeruginosa*-derived pyocyanin induces promising apoptotic and necrotic activities against MCF-7 human breast adenocarcinoma. *Microbial Cell Factories*, *21*(1), 262. https://doi.org/10.1186/s12934-022-01988-x
2. Abdelaziz, A. A., Abo Kamer, A. M., Al-Monofy, K. B., & Al-Madboly, L. A. (2022). A purified and lyophilized *Pseudomonas aeruginosa* derived pyocyanin induces promising apoptotic and necrotic activities against MCF-7 human breast adenocarcinoma. *Microbial Cell Factories*, *21*(1), 262. https://doi.org/10.1186/s12934-022-01988-x
3. Alley, M. C., Scudiero, D. A., Monks, A., Hursey, M. L., Czerwinski, M. J., Fine, D. L., Abbott, B. J., Mayo, J. G., Shoemaker, R. H., & Boyd, M. R. (1988). Feasibility of drug screening with panels of human tumor cell lines using a microculture

tetrazolium assay. *Cancer Research*, *48*(3), 589–601.
4. Alley, M. C., Scudiere, D. A., Monks, A., Czerwinski, M., Shoemaker, R. H., & Boyd, M. R. (1986). Validation of an automated microculture tetrazolium assay (MTA) to assess growth and drug sensitivity of human tumor cell lines. *Proceedings of the American Association for Cancer Research*, *27*, 389.
5. Al-Tememe, T. M. K., & Abbas, B. A. (2022). Molecular detection and phylogenetic analysis of *Pseudomonas aeruginosa* isolated from some infected and healthy ruminants in Basrah, Iraq. *Archives of Razi Institute*, *77*(2), 525–532. https://doi.org/10.22092/ARI.2022.357802.2099
6. Almami, I. S. (2025). Genetic mutations enhance the production of Exotoxin A by *Pseudomonas aeruginosa* for use as a potential anticancer therapeutic agent. *Experimental and Therapeutic Medicine*, *30*(2), 161. https://doi.org/10.3892/etm.2025.12911
7. ATCC. (n.d.). *MTT cell proliferation assay instruction guide*. American Type Culture Collection. www.atcc.org
8. Bogiel, T., Depka, D., Rzepka, M., Kwiecińska-Piróg, J., & Gospodarek-Komkowska, E. (2021). Prevalence of the genes associated with biofilm and toxins synthesis amongst the *Pseudomonas aeruginosa* clinical strains. *Antibiotics*, *10*(3), 241. https://doi.org/10.3390/antibiotics10030241
9. Chang, H., Huang, H., Huang, T., Yang, P., Wang, Y., & Juan, H. (2013). Flow cytometric detection of reactive oxygen species. *Bio-protocol*, *3*(8), e431. http://www.bioprotocol.org/e431
10. Conice, M., & Usha, M. S. (2017). Purification of toxin from *E. coli* isolate Pe68 by ion-exchange chromatography. *Journal of Applied Science and Engineering Methodologies*, *3*(1), 440–446.
11. Duong-Ly, K. C., & Gabelli, S. B. (2014). Salting out of proteins using ammonium sulfate precipitation. In *Methods in Enzymology* (Vol. 541, pp. 85–94). Academic Press. https://doi.org/10.1016/B978-0-12-420119-4.00007-0
12. Ema, F. A., Shanta, R. N., Rahman, M. Z., Islam, M. A., & Khatun, M. M. (2022). Isolation, identification, and antibiogram studies of *Escherichia coli* from ready-to-eat foods in Mymensingh, Bangladesh. *Veterinary World*, *15*(6), 1497–1505.
13. Gerlier, D., & Thomasset, N. (1986). Use of MTT colorimetric assay to measure cell activation. *Journal of Immunological Methods*, *94*(1–2), 57–63.
14. HiMedia Laboratories. (n.d.). *CCK003 product instruction guide*. http://himedialabs.com/TD/CCK003.pdf
15. Horwitz, R., & Webb, D. (2003). Cell migration. *Current Biology*, *13*(21), R756–R759.
16. Hoyt, K. M., Barr, J. R., Hopkins, A. O., Dykes, J. K., Lúquez, C., & Kalb, S. R. (2024). Validation of a clinical assay for botulinum neurotoxins through mass spectrometric detection. *Journal of Clinical Microbiology*, *62*(6), e01629-23. https://doi.org/10.1128/jcm.01629-23
17. Ismaieel, M. S., Hassan, M. H., & Flayyih, M. T. (2025). Cytotoxic effects of ExoT toxin on MCF-7 and PC3 cells. *Egyptian Journal of Medical Microbiology*, *34*(2), 435–439.
18. Jawad, L. Q., & Rasheed, H. A. R. R. (2022). Isolation and purification of anticancer protein exotoxin A from *Pseudomonas aeruginosa*. *Iraqi Journal of Agricultural Sciences*, *53*(1), 48–56. https://doi.org/10.36103/ijas.v53i1.1507
19. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680–685. https://doi.org/10.1038/227680a0
20. Liang, C. C., Park, A. Y., & Guan, J. L. (2007). In vitro scratch assay: A convenient and inexpensive method for analysis of cell migration in vitro. *Nature Protocols*, *2*(2), 329–333. https://doi.org/10.1038/nprot.2007.30
21. Marey, M. A., Abozahra, R., El-Nikhely, N. A., Kamal, M. F., Abdelhamid, S. M., & El-Kholy, M. A. (2024). Transforming microbial pigment into therapeutic revelation: Extraction and characterization of pyocyanin from *Pseudomonas aeruginosa* and its therapeutic potential as an antibacterial and anticancer agent. *Microbial Cell Factories*, *23*(1), 174. https://doi.org/10.1186/s12934-024-02438-6
22. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1–2), 55–63. https://doi.org/10.1016/0022-1759(83)90303-4
23. Pharmacia Fine Chemicals. (1970). *Sephadex: Gel filtration in theory and practice*. Pharmacia Fine Chemicals.
24. Qin, Z., Zhang, H., Wu, Q., Wei, B., Wu, R., Guo, X., Xiao, H., & Wu, W. (2022). Glucose-dependent insulinotropic polypeptide and substance P mediate emetic response induction by masked trichothecene deoxynivalenol-3-glucoside through signaling. *Toxins*, *14*(6), 371. https://doi.org/10.3390/toxins14060371
25. Saleh, R. H., Naher, H. S., & Al-Saadi, M. A. K. (2012). Molecular investigation of type III secretion system toxins-encoding genes in clinical isolates of *Pseudomonas aeruginosa*. *Medical Journal of Babylon*, *9*(4), 858–867.
26. Thabit, A. G., Sediek, M. N., & Mohamed, M. S. E. (2025). The presence of exotoxin genes and biofilm production in carbapenem-resistant *Pseudomonas aeruginosa* clinical isolates. *Germs*, *15*(1), 26–36. https://doi.org/10.18683/germs.2025.1452

27. Van Meerloo, J., Kaspers, G. J., & Cloos, J. (2011). Cell sensitivity assays: The MTT assay. *Methods in Molecular Biology*, *731*, 237–245. https://doi.org/10.1007/978-1-61779-080-5_20
28. Veetilvalappil, V. V., Manuel, A., Aranjani, J. M., Tawale, R., & Koteshwara, A. (2022). Pathogenic arsenal of *Pseudomonas aeruginosa*: An update on virulence factors. *Future Microbiology*, *17*(6), 465–481. https://doi.org/10.2217/fmb-2021-0158