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CHARACTERISATION OF AZURIN FROM *PSEUDOMONAS AERUGINOSA* AND ASSESSMENT OF ITS CYTOTOXICITY

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ABSTRACT:
Pseudomonas aeruginosa is a Gram-negative bacterium that is known for its virulence. The organism's exotoxins are major contributors to its virulence. This study details the characterisation and cytotoxicity of the azurin toxin purified from *Pseudomonas aeruginosa* isolated from the street vegetable salad sample. The band excised after the SDS-PAGE post-purification was subjected to MALDI-TOF analysis for protein identification. The excised band was approximately around 14 kDa. MALDI-TOF-based protein identification identified the protein as azurin with a score of 96, along with 59% protein sequence coverage. The purified toxin P2 was tested for its cytotoxicity and exhibited moderate, dose-dependent anticancer activity against Caco-2 colon cancer cells, with an IC_{50} of approximately 588 µg/mL. P2 exhibited minimal cytotoxicity towards HDF cells by maintaining more than 75% viability.

KEYWORDS: *Pseudomonas aeruginosa*, azurin, SDS-PAGE, MALDI-TOF, cytotoxicity.

INTRODUCTION

Pseudomonas aeruginosa is a common bacterium that primarily affects immunocompromised individuals and causes nosocomial infections (Tawfiq, 2018). This Gram-negative bacterium is non-sporulating, motile, and has a polar flagellum; it is an opportunistic pathogen that can cause infections such as sepsis, UTIs, lung infections, and infections of the skin, bone, and joints (Aljarah, 2018; Al-Saffar & Jarallah, 2019).

Water activity, suitable pH, and nutrients promote the growth of opportunistic pathogens like *Pseudomonas aeruginosa* on fresh produce. This raises the risk of contamination and foodborne illness. The pathogen's ability to cause disease depends mainly on the expression of virulence genes such as exotoxin A (toxA), type III secretion system genes (exoS, exoT, exou, and exoY), and elastases (lasA and lasB). These factors damage cells by disrupting cellular structures, hindering protein synthesis, and degrading extracellular matrix components. PCR-based molecular detection of exotoxin A has previously been performed to evaluate its role in pathogenicity. (Chinmayi & Usha, 2026).

Bacterial proteins and peptides have recently come into the limelight as an important area of research because of their diverse biological activity and pharmacological applications. Several bacterial toxins have exhibited antimicrobial, anticancer, and immunomodulatory properties, making them an alternative to conventional methods. Among these bacterial toxins, azurin produced by *Pseudomonas aeruginosa* has emerged as a promising candidate for anticancer activity because of its diverse structural characteristics and ability to interact with cellular proteins (Huang et al., 2020; Yaghoubi et al., 2020).

Cancer presents a major health risk, reducing quality of life and causing death. Conventional chemotherapy drugs tend to be highly toxic and lack selectivity, affecting both cancerous and healthy cells, which often leads to severe side effects. This requires developing new, more effective anticancer drugs with fewer side effects to achieve the best possible treatment results. (Juthani et al., 2024; Siegel et al., 2016; Huang et al., 2020).

Azurin, as a toxin/protein, shows promising anticancer activity, mainly against breast cancer, by interfering with different signalling pathways (Unver et al., 2022). This selectivity arises from azurin's ability to infiltrate cancer cells, inducing cytotoxicity and apoptosis while remaining non-toxic to normal cells. Emerging chemotherapeutic strategies involve using bacteria and their metabolites to treat cancer (Gao et al., 2017; Zhang et al., 2017; Yu et al., 2016).

An important aspect of a toxin to be considered for a therapeutic evaluation is not only its anticancer property but also its effect on non-malignant cells. Therefore, a comparative assessment using both models is important, as it provides preliminary information about the toxin's differential sensitivity. (Huang et al., 2020; Yaghoubi et al., 2020).

Recent studies continue to demonstrate azurin's cytotoxic effects. Zaghloul et al. (2025) isolated native azurin from a *Pseudomonas aeruginosa* strain and characterised the protein before testing its antimicrobial or anticancer activity. This research offers recent evidence confirming the biological activity of naturally sourced azurin purified from *Pseudomonas aeruginosa* and highlights the need to assess its effects on both malignant and non-malignant cells when exploring its potential for biomedical use (Zaghloul et al., 2025).

This study aims to characterise purified azurin using MALDI-TOF. The toxin's cytotoxicity was tested on normal human dermal fibroblasts (HDFs), and its anticancer activity was evaluated in human colorectal adenocarcinoma (Caco-2) cells to explore its potential as a selective therapeutic bioactive protein.

MATERIALS AND METHODS

Source and preparation of the protein: The protein sample was obtained from the cell-free supernatant of *Pseudomonas aeruginosa* isolate (S07VO-1) used in the present study, which was isolated from a street vegetable sample (Fig. 1). After production protein, the extracellular proteins were precipitated by 80% ammonium sulphate precipitation. After recovery of the precipitated protein, it was subjected to the gel filtration technique for purification using Sephadex-50. The purified protein was run on SDS-PAGE, excised, and subjected to MALDI-TOF analysis.

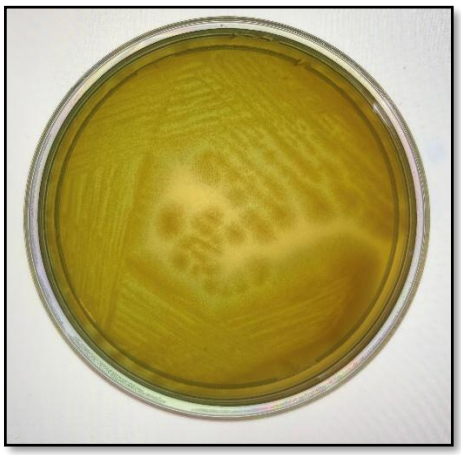


Fig. 1: Growth of *Pseudomonas aeruginosa* on King's B medium

Characterisation of toxin using MALDI-TOF analysis: The protein band representing the target protein was excised and sent 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 mixed with an equal volume of 2,5 dihydroxybenzoic acid (DHB) matrix and subsequently spotted onto the MALDI target plate. The matrix facilitated peptide desorption and ionisation upon laser exposure. The mass spectrum was acquired in positive ion reflectron mode using FlexControl software, ensuring accurate peptide mass measurements. The resulting peptide mass fingerprint was analysed through the SwissProt database using the Mascot search engine, with trypsin set as the digestion enzyme. Protein identification was based on Mascot score evaluation, matching peptide masses, and assessment of statistical significance.

Cytotoxicity study: The study was carried out using an MTT assay with a Human colorectal adenocarcinoma cell line and a human dermal fibroblast cell line.

Maintenance of cell lines: The Caco-2 cell line (Human colorectal adenocarcinoma) was obtained from the National Centre for Cell Science (NCCS), Pune, India. Cells were cultured in MEM supplemented with NEAA, 10% FBS, and 1% antibiotic-antimycotic solution, and kept in a CO2 incubator at 37 °C with 5% CO2 and 18-20% O2. They were subcultured every 2 days, and passage number 39 of Caco-2 cells was used for the experimental study (Freshney, 2016)

The HDF (Human dermal fibroblast cell line) was purchased from Himedia Labs (Cat No: CL005, Himedia), Mumbai, India. The cells were maintained in DMEM with high glucose supplemented with 10 % FBS along with the 1% antibiotic-antimycotic solution in an atmosphere of 5% CO2, 18-20% O2 at 37°C in the CO2 incubator and subcultured every 2 days. Passage number 11 of HDF was used for the present study.

Once the cell density reached 80%, cells were detached with a solution of 0.025% trypsin and 0.01% EDTA in D-PBS. Cell viability and counts were then assessed using a hemocytometer. Cells were then seeded at an appropriate density into T25 flasks and cultured until needed for cell-based assays (Freshney, 2016).

Cell viability and proliferation studies: MTT assay: The cytotoxic effects of the test compound P2 were assessed on Caco-2 and HDF cells using the MTT assay. MTT, a tetrazolium salt, is reduced by mitochondrial lactate dehydrogenase to insoluble purple formazan crystals. Briefly, cells were plated at 15,000 per 200µl of suitable media in a 96-well plate and incubated overnight at 37°c. After cells adhered to the surface of the culture plate, the spent medium was replaced with complete medium containing different concentrations of Test compounds at 15.62, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL. Cisplatin (10 µg/mL) was used as the reference standard. After adding the drug, cells were incubated for 24 hours at 37°C with a 5% CO2 atmosphere. After incubation, 100 µl of MTT solution (0.5 mg/mL) was added, and the cells were incubated for an additional 3 hours at 37 °C. DMSO (100 µl) was then used to dissolve the formazan crystals, and the purple color intensity was measured at 570 nm with a microplate reader (ELX-800, BioTek, USA). Cell viability was set to 100% for cells treated with only DMEM. Percentage cell viability was calculated using the following formula:

% cell viability = [OD of treated cells/OD of Untreated cells] * 100

The IC50 value was determined using the linear regression equation, i.e., Y = Mx + C. Here, Y = 50, and M and C were derived from the viability graph (Mosmann, 1983; van Meerloo et al., 2011).

Cell morphological assessment: Morphological changes in Caco-2 and HDF cells were assessed after treatment with test compounds at concentrations ranging from 15.62 ug/ml to 500 ug/ml for 24 hours at 37 °C. Cells were observed and recorded using an inverted biological microscope (CKX-41, Olympus) with a camera, and images were captured using MICAM software at 20x magnification. Scale: 100 µm (van Meerloo et al., 2011).

RESULTS

Characterisation of toxin using MALDI-TOF: The MALDI-TOF peptide mass fingerprinting found Azurin with a Mascot protein score of 96, exceeding the significance threshold (70). The protein was identified with an E-value of 0.00015 and 59% sequence coverage. Five representative peptide ions at m/z values 4537.2100, 1108.5300.1352.7900, 1681.8600, 1238.6600 matched the theoretical peptides K. QFTVNLSHPGNLPKNVMGHNWVLSTAADMQGVVTDGMASGLDK.D, K. DYLKPDDSR.V, R. VIAHTKLIGSGEK.D, K. LIGSGEKDSVTFDVSK.L and K. DSVTFDVSKLK.E, respectively. These peptide matches, together with the overall Mascot score, support the identification of azurin (Fig. 2-5).

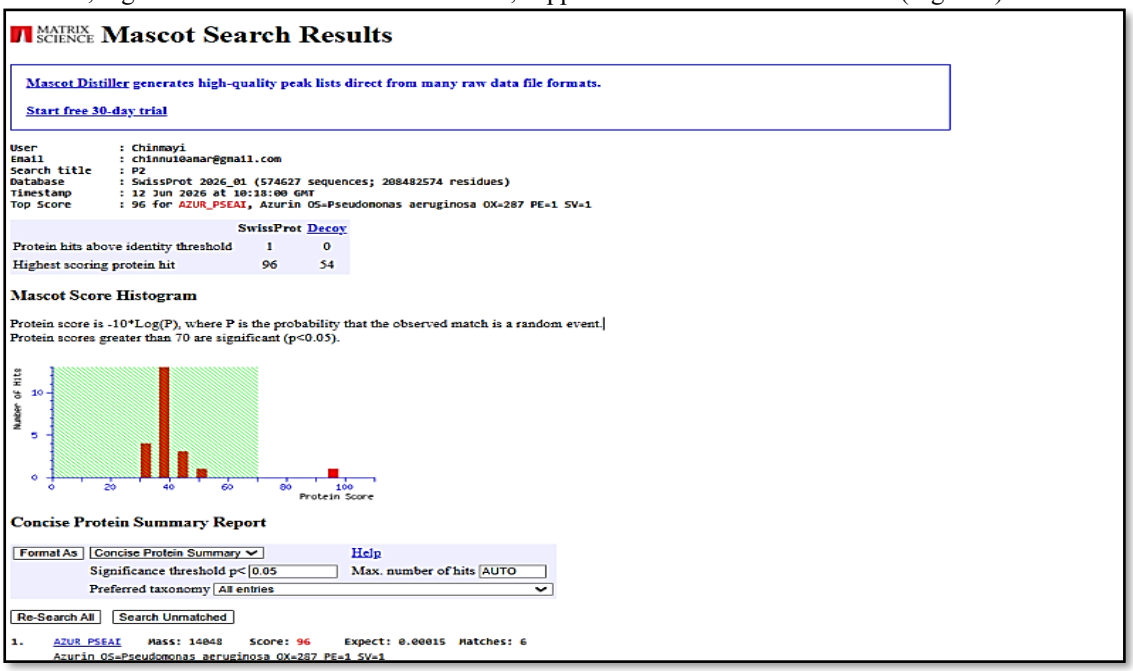


Fig 2: MASCOT search results with histogram and strain identification for the *Pseudomonas aeruginosa* isolate S07VO-1 toxin azurin

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

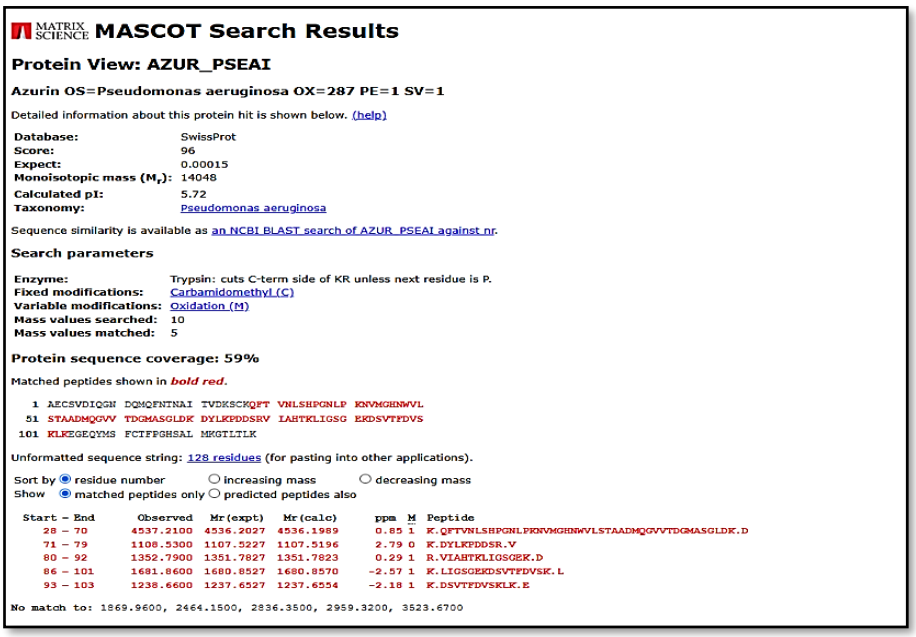
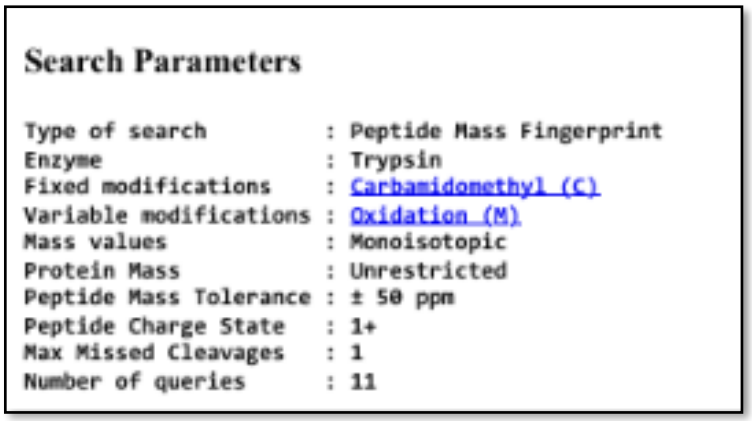


Fig 3: MASCOT search results with molecular weight and protein sequence coverage for azurin toxin from the *Pseudomonas aeruginosa* isolate S07VO-1



Search Parameters

Type of search	: Peptide Mass Fingerprint
Enzyme	: Trypsin
Fixed modifications	: Carbamidomethyl (C)
Variable modifications	: Oxidation (M)
Mass values	: Monoisotopic
Protein Mass	: Unrestricted
Peptide Mass Tolerance	: ± 50 ppm
Peptide Charge State	: 1+
Max Missed Cleavages	: 1
Number of queries	: 11

Fig 4: Mascot database search parameters used for protein identification

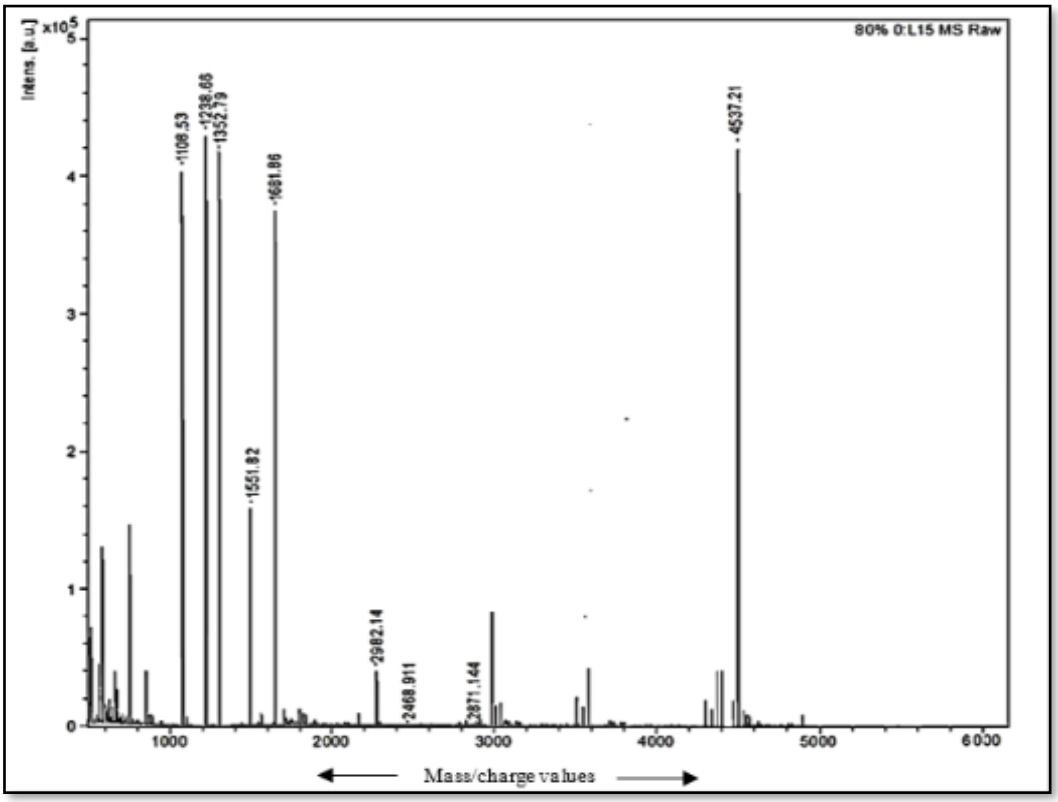


Fig 5: MALDI-TOF spectrum used to identify the *Pseudomonas aeruginosa* protein (P2) associated with azurin toxin

Cytotoxicity studies: The cytotoxicity of compound P2 was assessed on Caco-2 (human colon cancer) and HDF (normal human dermal fibroblasts) cells via an MTT cell viability assay following 24 hours of treatment. P2 exhibited a concentration-dependent reduction in cell viability in Caco-2 cells. Untreated cells showed 100% viability, while exposure to the positive control, cisplatin (10 µg/mL), decreased viability to 66.57%, demonstrating the assay system's responsiveness. Exposure of Caco-2 cells to P2 at concentrations ranging from 15.62 to 500 µg/mL gradually decreased viability from 98.93% to 60.70%. The decrease in cell viability became more evident as concentrations increased, indicating a dose-dependent cytotoxic effect. The calculated IC_{50} value was 588 µg/mL after 24 hours of treatment.

In Human Dermal Fibroblast (HDF) cells, untreated HDF cells exhibited 100% viability, whereas cisplatin treatment significantly reduced viability to 31.70%, demonstrating the expected cytotoxic effect of the positive control. In contrast, P2 treatment showed minimal toxicity to HDF cells across all tested concentrations. Cell viability remained above 90% up to 62.5 µg/mL and above 75% even at the highest tested concentration of 500 µg/mL. Specifically, viability decreased gradually from 97.63% at 15.62 µg/mL to 75.69% at 500 µg/mL. Since cell viability did not drop below 50% across the tested concentration range, an IC_{50} value could not be determined (reported as NA). These results show that P2 has minimal cytotoxic effects on normal fibroblast cells and remains highly compatible with cells even at higher concentrations (Fig. 6-9) (Tables 1 and 2).

Table 1: Percentage cell viability of Caco-2 cells treated with various concentrations of P2 along with the IC_{50} value determined after 24 hours of treatment.

MTT Assay Summary: P2 vs Caco-2		
Drug conc (µg/mL)	**% cell viability**	**IC50 conc (µg/mL)**
Untreated	100	588
Cisplatin-10 µg	66.57	
P2-15.62 µg	98.93	
P2-31.25 µg	92.09	
P2-62.5 µg	84.58	
P2-125 µg	73.39	
P2-250 µg	68.84	
P2-500 µg	60.70	

Table 2: Percentage cell viability of HDF cells treated with various concentrations of P2 along with the IC_{50} value determined after 24 hours of treatment.

MTT Assay Summary: P2 vs HDF		
Drug conc (µg/mL)	**% cell viability**	**IC50 conc (µg/mL)**
Untreated	100	NA
Cisplatin-10 µg	31.70	
P2-15.62 µg	97.63	
P2-31.2 µg	96.38	
P2-62.5 µg	92.16	
P2-125 µg	85.16	
P2-250 µg	81.75	
P2-500 µg	75.69	

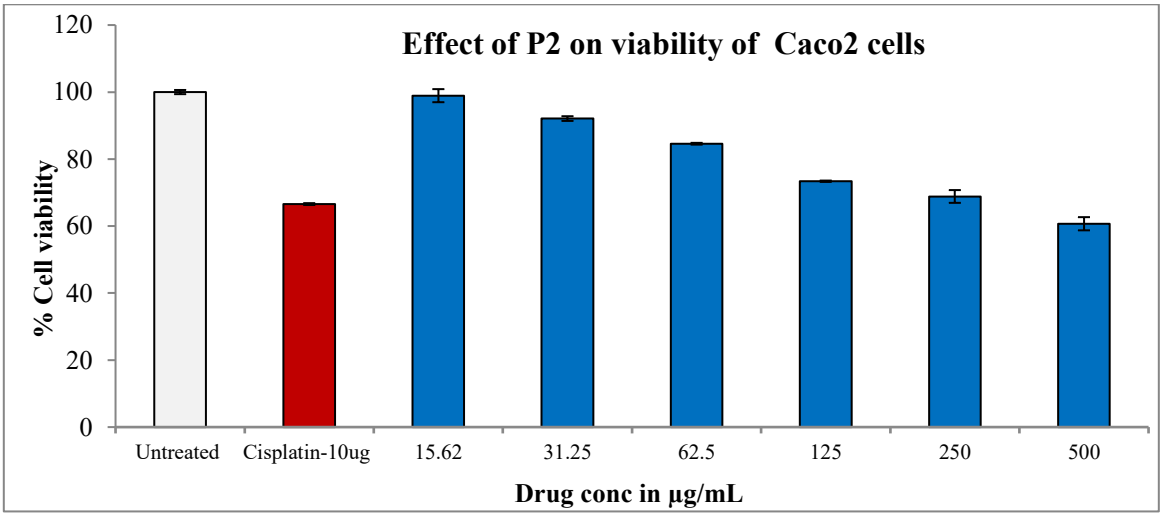


Fig 6: Bar graph showing the percentage of cell viability in Caco-2 cells treated with different concentrations of P2 after 24 hours of incubation.

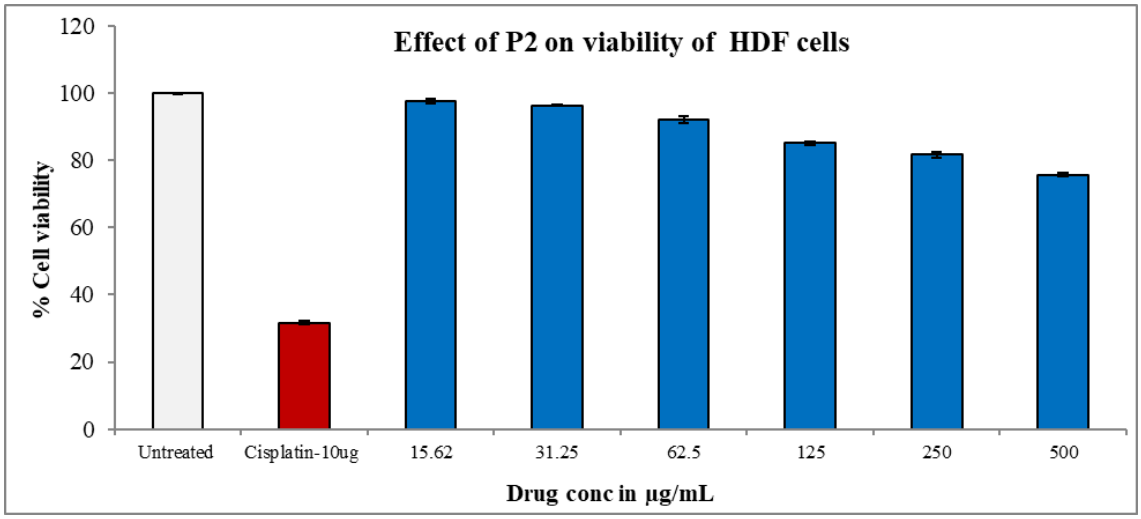


Fig 7: Bar graph showing the percentage of cell viability in HDF cells treated with different concentrations of P2 after 24 hours of incubation.

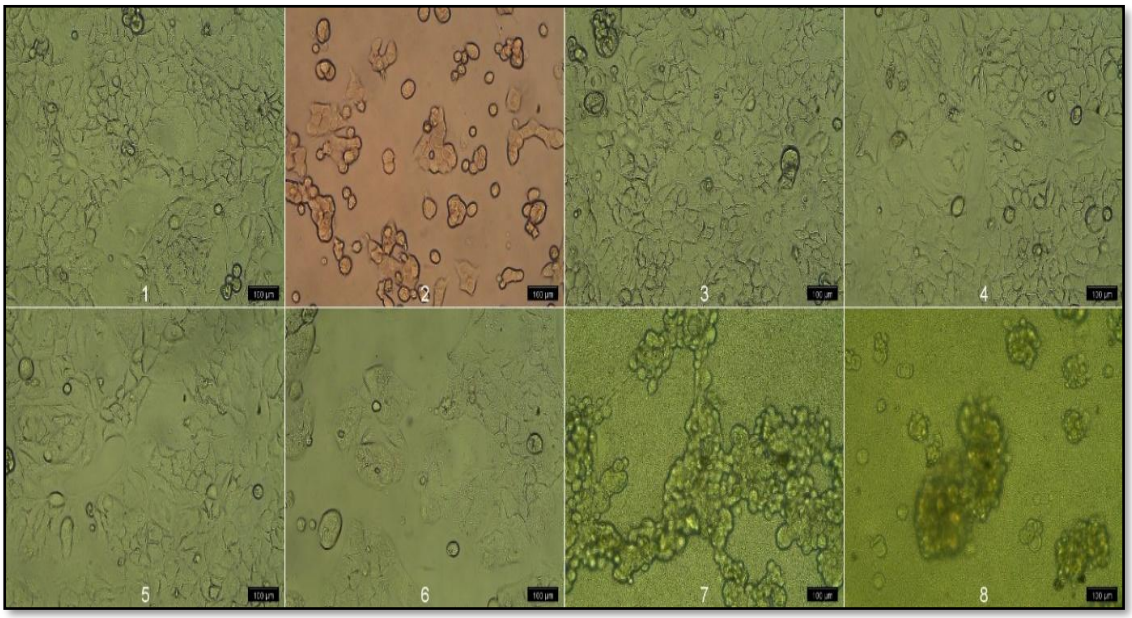


Fig 8: Overlaid montage images showing the morphology of Caco-2 cells treated with varying concentrations of P2 after 24 hours of incubation. All images were captured at 20× magnification using an inverted biological microscope and recorded with a digital camera equipped with MICAM software.
Legend: 1-Untreated, 2-Cisplatin-10µg/mL, 3-8: P2 at varying concentrations (15.62 µg/mL to 500µg/mL)

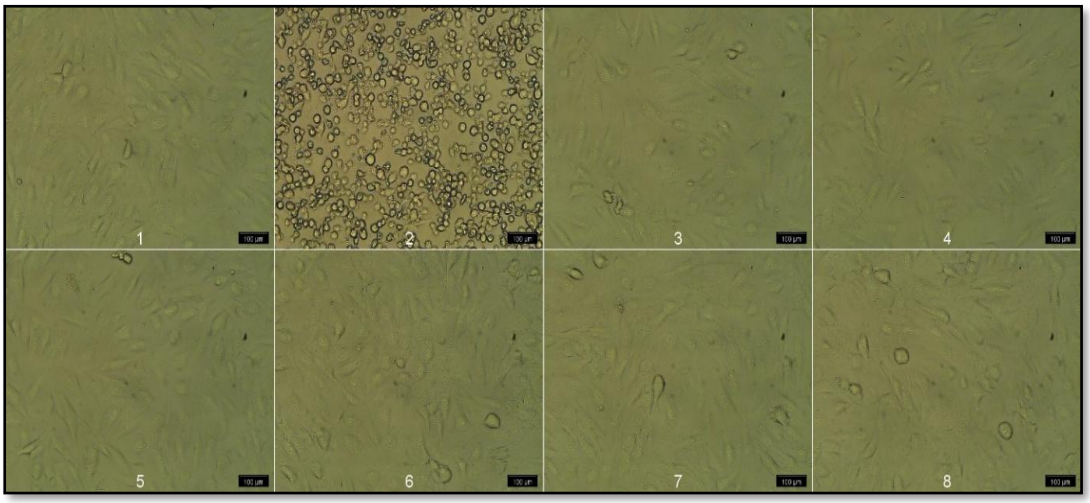


Fig 9: Overlaid montage images showing the morphology of HDF cells treated with varying concentrations of P2 after 24 hours of incubation. All images were captured at 20× magnification using an inverted biological microscope and recorded with a digital camera equipped with MICAM software.

Legend: 1-Untreated, 2-Cisplatin-10 µg/mL, 3-8: P2 at varying concentrations (15.62 µg/mL to 500 µg/mL)

DISCUSSION

The present study revealed that purified P2 from *Pseudomonas aeruginosa* was identified by MALDI-TOF PMF as azurin, with 59% sequence coverage, a Mascot protein score of 96, and 5 matched peptides.

Doellinger et al. (2020) discussed a MALDI-ToF MS study that identified cereulide from Bacillus cereus cultures. Cereulide was found in the ethanol wash solution, with peaks at m/z 1175 and 1191, confirming the presence of Bacillus cereus.

Manhique et al. (2020) investigated the occurrence of Enterobacteriaceae in ready-to-eat salads, water, and surface samples from markets in Maputo. Using MALDI-TOF MS, they rapidly identified 222 bacterial isolates from these sources. The most frequently identified species were Enterobacter cloacae, Klebsiella pneumoniae, and Enterobacter asburiae, while Escherichia coli was rarely found. This study demonstrates that MALDI-TOF MS is highly effective for accurate species-level identification of food-associated Enterobacteriaceae.

This study compared the activity of P2 in 2 cell lines: Caco-2 cells and HDF cells. At the maximum dose (500 µg/mL), Caco-2 cell viability decreased to 60.70%, whereas HDF cells remained at 75.69%.

The study conducted by Drigo et al. (2021) showed that MALDI-TOF MS proved effective for analysing bacterial toxins by identifying specific peptide markers linked to active botulinum neurotoxins. BoNT/C displayed distinctive peaks at m/z 1870.9 and 1059.5, while BoNT/D showed peaks at m/z 4013.5 and 1217.3. The approach achieved excellent diagnostic accuracy, with 100% sensitivity and 96.08% specificity, confirming the reliability of MALDI-TOF-based methods in toxin identification.

Kesik Oktay et al. (2026) investigated the cytotoxic effects of parasporal proteins from six native Bacillus thuringiensis isolates on various human cancer cell lines. The cancer cell lines used in the study included HeLa cervical cancer, MCF-7 breast cancer, A549 lung cancer, PC-3 prostate cancer, and Burkitt lymphoma cells. HEK-293 cells were used as a non-cancerous control to evaluate selectivity. The proteins were tested at 20 µg/mL for 48 hours, and cell viability was evaluated by MTT assay. Particularly, Bt 5.4 and Bt 5.10 significantly decreased cancer cell viability without affecting HEK-293 cells. Mainly, Bt 5.4 reduced A549 viability to about 51%, while Bt 5.10 and Bt 2.1 decreased HeLa cell viability to approximately 62% and 67%, respectively. Both Bt 5.4 and Bt 5.10 also demonstrated activity against MCF-7 cells. Due to its efficacy and minimal toxicity, Bt 5.4 was chosen for further testing.

Pahle et al. (2021) studied the cytotoxic activity of Clostridium perfringens enterotoxin (CPE) against different human pancreatic cell lines. Dose-dependent reduction in cell viability was seen with recombinant CPE, which expressed CPE receptors (Cldn3/4). After a 72-hour study, viability in BxPC-3 cells was reduced by 40% and 32 % in MIA PaCa-2 cells, whereas the cells that lack the Cldn3/4 receptors, like SK-MEL-5 cells, remained largely unaffected. PA-TU-8902 cells retained 60% viability and were less sensitive even at the highest CPE concentration. This difference shows that the response to CPE was affected not just by claudin levels but also by receptor accessibility. A similar response was observed when a translation-optimised CPE construct (optCPE) was introduced to pancreatic cancer cells. Resulting in approximately 40–85% cytotoxicity in most Cldn3/4-positive cell lines, whereas Cldn3/4-negative SK-MEL-5 cells remained unaffected. Moreover, silencing Cldn3/4 significantly reduced the cytotoxic activity, suggesting that CPE activity depended on its interaction with the receptors.

A study conducted by Haghighatafshar et al. (2023) demonstrated the cytotoxic effect of recombinant LukS-PV on MCF7 breast cancer cells and HEK293 cells as non-cancer cell lines for comparison. The tested protein showed high toxicity against the cancer cell lines at varying concentrations after 24 hours. The IC_{50} value for MCF7 cells was 49.64 ± 7.06 µg/mL, and HEK293 cells needed a higher concentration of 213.56 ± 14.62 µg/mL to exhibit the same inhibition. After 48 hours, the IC_{50} for MCF7 cells was 45.35 ± 5.38 µg/mL, whereas HEK293 cells needed 226.95 ± 8.41 µg/mL. The results show that recombinant LukS-PV exhibited greater toxicity toward cancer cell lines. The recombinant LukS-PV was loaded onto the silver nanoparticles, which exhibited a considerable increase in its cytotoxic activity. The IC_{50} of AgNPs+LukS-PV against MCF7 cells diminished to 13.11 ± 0.70 µg/mL after 24 hours and subsequently to 6.17 ± 0.17 µg/mL after 48 hours. Similarly, the IC_{50} values for HEK293 cells were 96.18 ± 2.03 µg/mL and 88.18 ± 1.65 µg/mL. The cytotoxic response was accompanied by apoptosis. At the IC_{50} concentration of AgNPs+LukS-PV, 33.2% of MCF7 cells underwent apoptosis, which increased to 55.6% at twice that concentration. Apoptosis was also seen in HEK293 cells, but the required concentration differed significantly. After 24 hours, the IC_{50} was about 13 µg/mL for MCF7 cells, whereas in HEK293 cells, it was approximately 96 µg/mL. These observations supported the cytotoxic potential of LukS-PV, along with its selectivity and safety, which require further investigation before checking its therapeutic significance.

Conclusion: The characterisation of P2 using MALDI-TOF PMF identified P2 as azurin with 59% sequence coverage, along with a Mascot protein score of 96. A comparison between the two cell lines shows that Caco-2 cells are more affected by P2 treatment than HDF cells. At the highest dose (500 µg/mL), Caco-2 viability dropped to 60.70%, while HDF cells maintained 75.69%. This difference indicates that P2 is more selectively toxic to Caco-2 cells, with less impact on normal cells. Such selectivity is beneficial for biomedical, pharmaceutical, or nutraceutical use, as it suggests a wider safety margin and lower toxicity risk to healthy tissues.

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