JOURNAL OF CLINICAL AND BIOMEDICAL SCIENCES

ORIGINAL ARTICLE

OPEN ACCESS

Received: 24-03-2025
Accepted: 29-07-2025
Published: 31-12-2025

Citation: MM Seyedeh, K Soroush, A Atefeh, B Mahsa, J Hossein, I Arman. Assessment of Cytotoxicity and Induction of Apoptosis by Cytolysin-A in MCF-7 Human Breast Cancer Cell Line. 2025; 15(4):277-283. https://doi.org/10.58739/jcbs/v15i4.25.139

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Funding: None

Competing Interests: None

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Published By Sri Devaraj Urs Academy of Higher Education, Kolar, Karnataka

ISSN
Print: 2231-4180
Electronic: 2319-2453

Assessment of Cytotoxicity and Induction of Apoptosis by Cytolysin-A in MCF-7 Human Breast Cancer Cell Line

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Abstract

A protein called cytolysin A or ClyA, encoded by certain bacteria species, can cause cytotoxicity. Although the ClyA protein is not typically expressed at detectable levels in most *E. coli* strains, here it was successfully overproduced and purified by cloning the structural gene into a hns mutant strain. The cytotoxicity of the purified cytolysin was assessed on two MCF-7 cancer cell lines and HDF normal cell line using the MTT assay. Flow cytometry was employed to examine the cytolysin's ability to induce apoptosis in cancer cells. In addition, a Western blot analysis was carried out to evaluate the expression levels of P53, Bcl2, and Bax proteins. The results revealed that cytolysin exhibited dose-dependent and time-dependent toxicity towards cancer cells, while showing minimal toxicity against normal cells, indicating its selective action against cancer cells. Cytolysin had an IC50 value of 3.29 µg/ml against MCF-7 cancer cells and 12.6 µg/ml against HDF normal cells. Flow cytometry results further demonstrated that cytolysin induced apoptosis in cancer cells, evidenced by increased expression of p53 and BCL2, as well as decreased in Bax, in gene and protein levels. These findings underscore the potential of cytolysin as a targeted therapy for cancer, highlighting its selective cytotoxic effect on cancer cells.

Keywords: Cytolysin-A, Breast cancer, Cytotoxicity, Flowcytometry, Western blotting

1 Introduction

Cytolysin A (ClyA) is a 34-kDa pore-forming protein (PFP) produced by certain bacteria within the Enterobacteriaceae family, including *Escherichia coli* and *Salmonella enterica*. The Ludwig and Oscarsson research groups independently observed that overexpressing the SlyA regulatory protein from *Salmonella enterica* serovar Typhimurium in *E. coli* K-12 led to the identification of this toxin, which confers a hemolytic phenotype in the recombinant *E. coli* strain. Since its initial discovery, research has concentrated on elucidating the structure and function of ClyA, along with investigating the *clyA* gene—first detected in *E. coli* K-12. [1, 2] Although present across many *E. coli* strains, the *clyA* gene is disrupted in approximately half, exhibiting various patterns of inactivation due to insertions or deletions. [3-5] This disruption is notably more frequent in *E. coli* phylogroup B2 strains, such as those associated with enteropathogenic and

extraintestinal infections, while other phylogroups typically retain an intact *clyA* gene. [3]

ClyA has been found to have homologs in other pathogenic bacteria, like *Salmonella* and *Shigella*. [6, 7] In *Shigella* species, the *clyA* gene is often rendered nonfunctional due to inactivation by insertion sequence elements in *S. dysenteriae*, *S. boydii*, and *S. sonnei*, or by frameshift mutations in *S. flexneri*, similar to what is observed in *E. coli*. [8] *Salmonella* Typhi and *Salmonella* Paratyphi A strains have been found to have intact clyA gene copies. [9] However, the *clyA* gene is absent in other strains such as *Salmonella enterica* serovar Paratyphi B and C, various non-typhoidal serovars (e.g., Typhimurium, Enteritidis, Choleraesuis, Dublin, and Gallinarum), *Salmonella enterica* subsp. *arizonae*, and *Salmonella bongori*. This indicates that the *clyA* gene and its homologs are selectively conserved in certain Enterobacteriaceae family members.

ClyA has been widely studied for its role as a virulence factor. The structural analysis of ClyA through X-ray crystallography and electron microscopy reveals that it can be both a soluble monomer and a transmembrane pore, undergoing significant conformational changes. [10, 11] These insights have advanced the understanding of other α-pore-forming toxins (α-PFTs). [12, 13] A vesicle-mediated pathway is used to secrete ClyA from bacterial cells. [14] Notably, recent research suggests potential clinical applications for ClyA, including its use in vaccine development and cancer therapy, as recombinant ClyA can be displayed on bacterial cell surfaces or released by membrane vesicles.

Bacterial toxins are crucial for pathogenicity as virulence factors and have the potential to be used in biotechnology. [15, 16] The recognition of ClyA as a virulence factor has led to its increased use in biotechnology, where its applications are being examined more frequently. Significant advancements have been made in understanding ClyA's properties, including its function, activity, secretion methods, and structure, since its discovery over 25 years ago. However, more research is needed to fully understand its physiological roles and the effects it has on host cells. Although ClyA is a key virulence factor that can cause harm, it also offers practical applications due to their unique characteristics. Recent research has highlighted how ClyA can be used in developing cancer therapies, nanopores, and vaccines, demonstrating its potential. [17] Considering that breast cancer is one of the most common cancers and the need to provide new treatment strategies for this disease, the aim of this study was to evaluate the cytotoxic effect of ClyA on MCF-7 cell line.

2 Materials and Methods

2.1 Cloning and purifying of ClyA

To overexpress the ClyA protein, *E. coli* strains were transformed with pUC18 plasmid carrying the *clyA* gene -pYMZ80-. [18] These strains were cultured at 37°C with shaking in Luria–Bertani (LB) broth or on LB agar plates. Blood agar plates were prepared using 5% horse erythrocytes and Columbia agar base. Carbenicillin was used at a concentration of 50 µg/ml for antibiotic selection. The mollicute *Acholeplasma laidlawii* strain A-EF22 was grown in a BSA/tryptose medium with added fatty acids and cholesterol, which were incorporated into the cell membrane. Cell size and morphology were observed using a Zeiss phase-contrast microscope. ClyA was purified from *E. coli* pYMZ80 by resuspending a culture in A50 buffer, followed by PAGE in a Bio-Rad Prep Cell. Fractions were collected and analyzed for cytolytic activity using SDS-PAGE. Protein concentration and silver staining were done using Bio-Rad kits, and the N-terminal sequence was determined via Edman degradation. Rabbit antibodies against ClyA were raised using the protein overexpressed from pYMZ80, with the 34 kDa band used for immunization. Antiserum was affinity-purified and used in Western blotting at a dilution of 1:1000, employing an alkaline phosphatase-conjugated secondary antibody. To quantify ClyA in cell extracts, samples were run on SDS-PAGE and compared with protein standards, stained with Coomassie blue. About 1.5 µg of ClyA protein was loaded per lane for Western blotting.

2.2 Assessment of ClyA Cytotoxicity

The MTT assay was utilized to assess the cytotoxic effects of ClyA on the MCF-7 breast cancer cell line. For this experiment, 5×10^3 MCF-7 cells were seeded into each well of a 96-well plate and incubated for 24 hours. Following this initial incubation, the cells were treated with various concentrations of ClyA (0.9, 1.8, 2.7, and 3.6 µg/ml) and further incubated for 24, 48, and 72 hours. At each time point, the supernatant was removed, and 20 µl of MTT solution was added to each well, followed by a 4-hour incubation at 37°C. After incubation, the supernatant was discarded, and 100 µl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. The

absorbance at 570 nm was then measured using an ELISA plate reader to determine cell viability. To investigate the impact of ClyA on normal cells, the MTT assay was also performed Human dermal fibroblasts (HDF) cells. In this case, 10^5 HDF cells were seeded in each well of a 96-well plate with a total volume of 100 µl per well and incubated for 24 hours. The cells were then exposed to various concentrations of ClyA (0.9, 1.8, 2.7, and 3.6 µg/ml). Untreated cells served as a control group. The procedure for HDF cells was conducted similarly to that of the MCF-7 cells. The percentage of cell survival was calculated using the following formula:

$$\text{Cell survival\%} = \frac{\text{OD of treated cells}}{\text{OD of control cells}} \times 100$$

2.3 Apoptosis Assay in MCF-7 Cells Treated with ClyA

2.3.1 Acridine Orange/Propidium Iodide (AO/PI) staining

To evaluate apoptosis, MCF-7 breast cancer cells were exposed to IC50 concentrations of ClyA for 24, 48, and 72 hours. Cells were removed from the culture flask using trypsin after the incubation period and then centrifuged at 2700 rpm for 5 minutes. A uniform suspension was achieved by resuspending the cell pellet in 1 ml of phosphate-buffered saline (PBS).

To stain the cells, 10 µl of the cell suspension was mixed with 10 µl of acridine orange (AO) and 10 µl of propidium iodide (PI) and allowed to incubate for 5 minutes. The stained mixture was then placed on a slide and viewed under a fluorescent microscope. This dual staining method allowed for the differentiation of live, apoptotic, and necrotic cells by identifying changes in membrane integrity and DNA condensation. [19]

2.3.2 Flow Cytometry for Apoptosis Assessment

MCF-7 cells were seeded in 6-well plates and treated with ClyA at concentration of IC50 for 24,48 and, 72 hours. The cells were then rinsed with phosphate-buffered saline (PBS) and stained with propidium iodide (PI) for 30 minutes after treatment. The cells were removed from the plates and examined by flow cytometry to determine apoptosis, which is determined by the uptake of PI, which identifies cells that have compromised membrane integrity.

2.3.3 Western Blotting Quantification of p53, Bax and BCL2 Gene Expression

Cells were subjected to ClyA treatment at concentrations of IC50 for 48 hours. Cells were harvested and RNA was extracted in accordance with the manufacturer's instructions (QIAwave RNA Mini Kit, Qiagen) following treatment. RNA integrity was verified through agarose gel electrophoresis, and cDNA was synthesized from the RNA samples. Real-time PCR was conducted using a Bio-Rad system to quantify the expression of p53, Bax and BCL2 genes, with primers specified in Table. 1. To ensure PCR specificity, a melting temperature curve was analyzed. Relative gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method, with GAPDH as the reference gene.

Table 1: Sequence of primers used for Real time PCR experiments

	Genes primer sets	
GAPDH	F: 5′ TGCACCACCAACTGCTTAGC 3′	R: 5′ GGCATGGACTGTGGTCATGAG 3′
BCL2	F: 5′ ATCGCCCTGTGGATGACTGAGT 3′	R: 5′ GCCAGGAGAAATCAAACAGAGGC 3′
Bax	F: 5′ TCAGGATGCGTCCACCAAGAAG3′	R: 5′ TGTGTCCACGGCGGCAATCATC3′
P53	F: 5′ ACACGCTTCCCTGGATTGG 3′	R: 5′ GGTCTTGGCCAGTTGGCAA 3′

Proteins were extracted using RIPA Lysis Buffer (Merck) and quantified with the Bicinchoninic Acid (BCA) Protein Assay Kit (Beyotime). Equal protein amounts (30 µg per lane) were separated by SDS-PAGE and transferred to PVDF membranes (Beyotime). Membranes were blocked with 3% bovine serum albumin (BSA) (Sigma-Aldrich) for 1.5 hours and then incubated overnight at 4°C with primary antibodies. After washing, membranes were probed with goat anti-rabbit IgG-HRP secondary antibody (1:2000; Abcam) for 2 hours. Protein bands were detected using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) and visualized with X-ray film.

3 Results

3.1 Isolation and Characterization of Native ClyA Cytolysin

The clyA gene from *E. coli* K-12 was cloned into the pUC18 plasmid and expressed in the hns mutant strain pYMZ80. SDS-PAGE analysis showed that ClyA migrates at approximately 34 kDa. We developed a rapid purification method, using 6% cylindrical PAGE. Hemolytic activity was observed in specific fractions, which were pooled and analyzed. Coomassie blue staining revealed a predominant 34 kDa band, while silver staining detected additional faint bands that did not correlate with hemolytic activity.

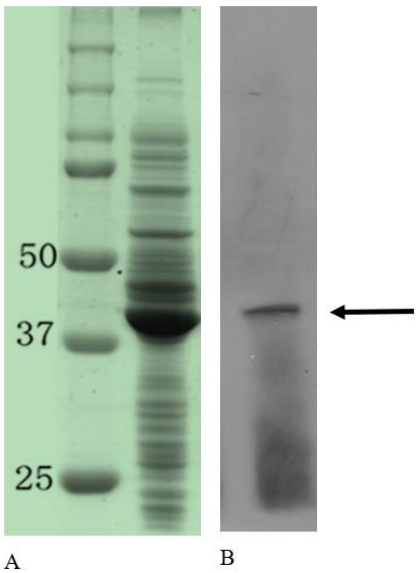

Fig. 1: A. Expression of cloned ClyA. Coomassie Blue-stained SDS–PAGE (15%) analysis of polypeptides derived from the strains. B. SDS–PAGE analysis of purified ClyA. A pool of selected fractions was subjected to SDS–PAGE (10%) and silver staining. The major protein band of approximately M_r 34000 representing ClyA is indicated with an arrow

3.2 Cytotoxicity of ClyA

The MTT assay revealed that ClyA caused significant and dose-dependent cytotoxicity in MCF-7 cells, with increased cell death correlating with higher concentrations and longer treatment times (Fig. 2-A, P <0.001). Conversely, normal HDF cells showed no significant cytotoxicity at lower ClyA doses, with noticeable cell death occurring only at the highest ClyA concentration (3.6 and 4.5 µg/ml) after 48 and 72 hours (Fig. 2-B). The IC-50 values for ClyA were 3.29 µg/ml for MCF-7 cancer cells and 12.6 µg/ml for HDF normal cells.

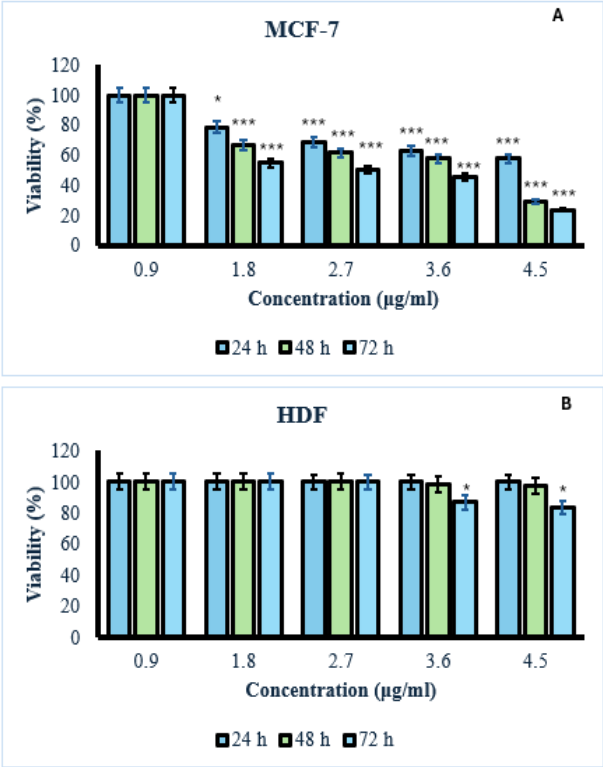

Fig. 2: Cytotoxicity of ClyA in (A) MCF-7 cancer cells and (B) HDF normal cells. The data demonstrate that these ClyA have a significant cytotoxic effect on cancer cells while showing minimal impact on healthy cells (*P < 0.05; ***P < 0.001)

3.3 Apoptosis Induced by ClyA

The apoptotic effects of ClyA on MCF-7 cells were analyzed using AO/PI staining, flow cytometry, and real-time PCR. Both staining and flow cytometry results showed that apoptosis increased with time (Fig. 3-A to H). Real-time PCR data indicated a significant overexpression of p53 and BCL2, as well as a downregulation of Bax (Fig. 3-I). These results suggest that the cytotoxicity of the ClyA is primarily due to their ability to induce apoptosis.

3.4 Western Blot Analysis

Western blotting was utilized to measure the expression levels of P53, BAX, and BCL2 proteins. Analysis with ImageJ software showed a reduction in BAX level, while P53 and BCL2 levels were notably elevated (Fig. 4).

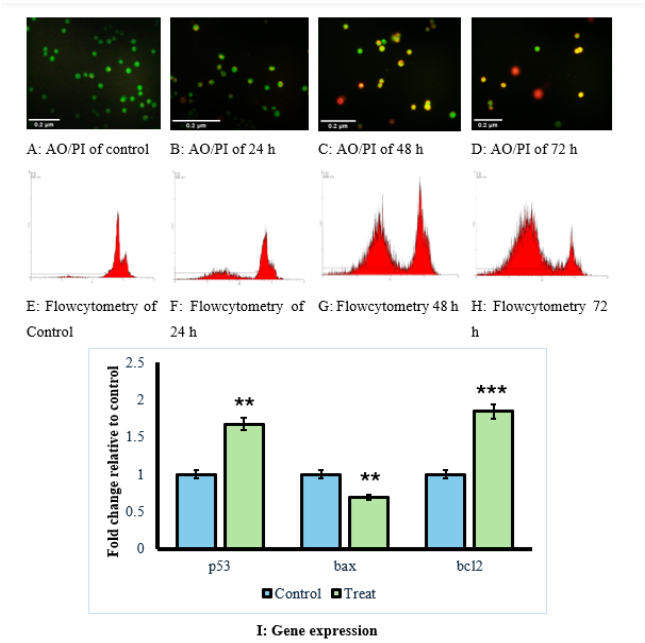

Fig. 3: Apoptosis induction in MCF-7 cells treated with ClyA. The figure shows: First row: AO/PI staining images, Second row: Flow cytometry results, Third row: Gene expression data. These methods confirm that apoptosis is induced in a time-dependent manner by the ClyA (*P < 0.01; ***P < 0.001)**

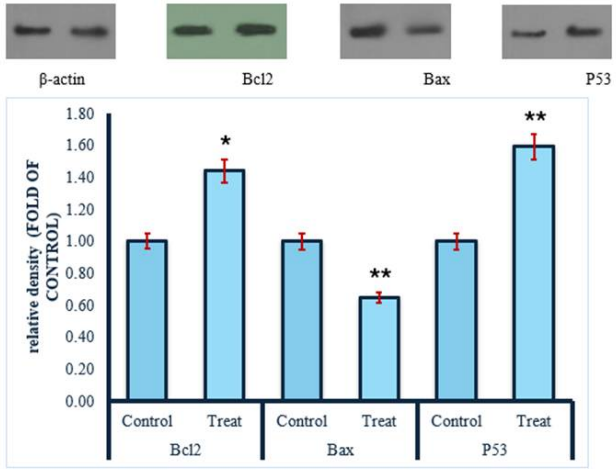

Fig. 4: Western blot analysis of signaling molecules in MCF-7 cells treated with ClyA. The results indicate downregulation of BAX proteins, and upregulation of P53 and BCL2 protein

4 Discussion

The identification of cytolytic properties in *E. coli* K-12 strains, traditionally regarded as non-pathogenic, has sparked significant interest in understanding the ClyA cytolysin's role and mechanism. Overproducing and purifying ClyA made it possible to conduct in-depth in vitro analyses, which confirmed its inherent cytolytic activity. In osmotic protection assays, it was discovered that ClyA produces pores in erythrocytes with diameters that range from 2.2 to 3.5 nm. Osmotic protection assays revealed that ClyA forms pores in erythrocytes with diameters ranging from 2.2 to 3.5 nm. [17] The specific activity of ClyA was found to be approximately 200 hemolytic units (HU) per mg on horse erythrocytes, which is comparable to the activity of delta-toxin from *Staphylococcus aureus* (200 HU per mg) as reported by Alouf *et al*. [20]

The use of bacterial products, which can include toxins, has been investigated historically as a means of fighting and treating cancer. A combination of heat-killed bacteria, known as Coley's toxin, was employed to trigger an anti-cancer immune response. This concept has led to the investigation of the ClyA protein and ClyA-OMV (outer membrane vesicle) system for their potential in cancer therapy. [17]

Bacteria produce valuable compounds that can be used for various purposes. [21, 22] One of these compounds are bacterial toxins. The wild strain can produce these toxins in very small amounts at times. Therefore, in the present research, the synthesis and purification of cytolysin were studied using cloning. The investigation focused on its cytotoxic effects on MCF-7 and HDF cell lines. According to the results, cytolysin has a specific cytotoxic effect on cancer cells. It can be concluded that cytolysin did not have a significant effect on healthy cells at the concentrations we examined. Cytolysin at a concentration equivalent to its IC50 was evaluated to induce apoptosis in cancer cells. Methods such as Acridine orange/propidium iodide staining, flow cytometry, evaluation of gene expression and pro-apoptotic and anti-apoptotic proteins were used for this purpose, which all showed that cytolysin induced apoptosis in cancer cells.

Cancer is still a major health challenge worldwide, leading to ongoing research into various treatment strategies. [15, 16] Studies have shown that ClyA can increase necrosis in tumors, facilitate the presentation of tumor antigens, and improve drug delivery to cancer cells. [23, 24] With advances in engineering techniques, ClyA-OMVs are being optimized for targeted therapies and immune response enhancement. This approach holds the

potential to accelerate research and develop more effective cancer treatments. [24]

Research by Ryan *et al*. [25] demonstrated that ClyA, combined with a hypoxia-inducible promoter, enhanced necrosis in tumor hypoxic regions and inhibited tumor growth in mice. This suggests ClyA's potential as an anti-tumor agent. *E. coli*-derived OMVs have shown promise in tumor immunotherapy. Intravenous administration of these OMVs elicited robust anti-tumor responses mediated by IFN-γ and T cells. [24] Additionally, OMVs have been engineered to present tumor-associated antigens, such as PD-1, to disrupt tumor cell interactions and reduce PD-1 ligand expression. Recent studies highlight the versatility of ClyA-OMV systems. Thomas et al. found that ClyA could anchor hyaluronidase (Hy) to OMVs produced by *E. coli* Nissle, facilitating targeted delivery of Hy to tumors. This approach enhanced drug penetration by reducing hyaluronic acid levels in tumor tissues. [26] ClyA has also been used to display therapeutic agents on OMVs. For instance, OMVs displaying affibodies against HER2 (a protein overexpressed in breast cancer) successfully delivered siRNA to HER2-positive ovarian and breast cancer cells, inducing cell death with minimal side effects. A novel approach utilizes ClyA as a fusion partner in a "plug-and-display" system to present tumor antigens on OMVs. [27] This method allows for the simultaneous display of multiple tumor antigens, which are effectively delivered to lymph nodes and presented to dendritic cells, triggering antigen-specific T-cell responses in murine models. [28] Overall, these advances suggest that ClyA and its associated technologies could significantly enhance cancer treatment through targeted cytolytic therapy, antigen delivery, and immune system modulation. [29]

ClyA's capacity to fuse with foreign proteins and anchor them on bacterial surfaces enhances its utility for displaying various prokaryotic and eukaryotic proteins. By using ClyA in combination with OMVs, this capability is further enhanced. Surface-exposed antigens facilitated by ClyA are more immunogenic than cytoplasmic ones, making them advantageous for vaccine development. [30, 31] In tumor therapy, ClyA serves as a fusion partner for tumor antigens and is integral to OMV-based delivery platforms that target specific cancer cells. [26, 29, 32] Although it has promising applications, there is still a lack of understanding of ClyA's biphasic activity and its physiological effects in vivo. ClyA has potential applications, but its biphasic activity and physiological effects in vivo remain unknown to the public. Future developments that incorporate ClyA, particularly in vaccine and cancer therapy, should carefully consider its potential side effects and inherent toxicity. [17]

Rho *et al*. examined the cytotoxic action of *Vibrio vulnificus* cytolysin on cultured pulmonary artery endothelial (CPAE) cells, revealing a series of cellular disruptions. The cytolysin notably increased cytosolic free Ca^{2+} levels, triggered DNA fragmentation, and lowered cellular concentrations of NAD^+ and ATP. Interestingly, these toxic effects were reduced when EGTA and aminobenzamide were applied, although verapamil and catalase showed no protective effect. The findings indicate that cytolysin-induced rises in cytosolic Ca^{2+} promote DNA fragmentation, which then activates nuclear poly(ADP-ribose) synthetase. This enzymatic activation results in NAD^+ and ATP depletion, ultimately leading to cell death. [33]

The results of this study indicated that cytolysin could be a promising compound in the tumor therapy. By inducing apoptosis, this bacterial toxin causes its cytotoxic effects. Genetic and metabolic engineering may assist in the production of cytolysin in high quantities, which is one of the challenges facing its use.

5 Declarations

Ethical Approval: This study was not on animal or human samples and did not require ethical approval.

Consent to Participate: This study was not on animal or human samples and did not require consent to participate.

Consent to Publish: This study was not on animal or human samples and did not require consent to publish.

Author Contribution: S.M.M designed research; performed research; contributed analytic tools; analyzed data; and reviewed the paper. S.K, A.A., M.B, H.J., and AI helped in designing the research, analyzed data, and wrote the paper.

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