IJMCM
International Journal of Molecular and Cellular Medicine
p-ISSN: 2251-9637 o-ISSN: 2251-9645
Babol University of Medical Sciences
Cellular and Molecular Biology Research Center

Anti-tumor Effects of Recombinant Clostridium α-Toxin on Breast Cancer: An *In Vitro* and *In Vivo* Study

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Article type:
Original Article

Received:
2024.08.08
Revised:
2024.10.05
Accepted:
2024.10.23

ABSTRACT

Cancer is the second leading cause of death worldwide, surpassed only by cardiovascular diseases. This study investigated the anticancer effects of recombinant *Clostridium* α-toxin on breast cancer, both *in vitro and in vivo*. The entire coding sequence of a codon-optimized *α-toxin* was designed, cloned into the pET28a (+) vector, and expressed as recombinant α-toxin in *Escherichia coli* (*E. coli*) BL 21(DE3) cells transformed with the recombinant plasmid. The recombinant α-toxin was purified using Ni^{2+} affinity chromatography, and its accuracy and purity were confirmed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. The anticancer effects of purified α-toxin were then assessed *in vitro* and animal models against MCF-7 breast cancer cells. Protein analysis confirmed the presence of a 48 kDa band corresponding to the recombinant α-toxin. Additionally, the IC_{50} values of α-toxin against MCF-7 cells at 24, 48, and 72 h were 407.3±2.392 µg/mL, 287.3±5.411 µg/mL, and 258.1±4.671 µg/mL, respectively. *In vivo*, results demonstrated a significant reduction in mean cancer nodule size following α-toxin treatment (p<0.001). These findings suggest that α-toxin may serve as a promising candidate for breast cancer therapy.

Keywords: α-toxin, *Clostridium*, breast cancer, *in vitro*, *in vivo*

Cite this article: Rezaei Khozani N, *et al*. Anti-tumor Effects of Recombinant Clostridium α-Toxin on Breast Cancer: An *In Vitro* and *In Vivo* Study. International Journal of Molecular and Cellular Medicine. 2024; 13(4):404-416. **DOI:** 10.22088/IJMCM.BUMS.13.4.404

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Introduction

Breast cancer is a complex and significant global health issue characterized by the uncontrolled proliferation of malignant cells within breast tissue (1). It remains one of the most prevalent cancers, posing substantial physical, emotional, and socioeconomic burdens on individuals diagnosed with the disease (2). Ongoing research continues to explore the intricacies of breast cancer, with a focus on improving detection, treatment, and survival outcomes (3).

Breast cancer treatment requires a multifaceted approach tailored to factors such as cancer stage, tumor characteristics, and the patient's overall health. A multidisciplinary team—comprising oncologists, surgeons, and radiation specialists—is essential in developing individualized treatment plans (4-6).

Emerging research highlights the potential role of bacteria in cancer therapy, either through direct anti-tumor mechanisms or as vectors for delivering therapeutic agents (7). Bacteria offer distinct advantages over conventional cancer treatments due to their unique biological properties (8). Firstly, many tumors present hypoxic conditions that favor the growth of anaerobic bacteria. Secondly, bacterial behavior can be effectively controlled, potentially addressing some limitations of traditional cancer therapies (9). Moreover, bacterial therapies exhibit superior penetration into tumor tissues compared to radiation and other modalities. However, challenges such as bacterial toxicity and genetic instability must be addressed. Advances in this field include the engineering of bacteria through recombinant DNA technology and the combination of bacterial therapy with chemotherapy, heat shock proteins, heavy metals, and radiation.

Microbial toxins, particularly those derived from bacterial species, are gaining attention as potential anticancer agents (10). These toxins, such as those produced by *Clostridium* and *Pseudomonas* species, have demonstrated the ability to disrupt cancer cell membranes, inhibit protein synthesis, and induce immune responses (11, 12). A notable bacterial toxin with specific activity against cancer cells is the α-toxin produced by *Clostridium septicum*.

C. septicum is an anaerobic, motile, spore-forming, toxin-producing Gram-positive bacillus commonly isolated from the human intestines and typically associated with spontaneous gas gangrene in individuals with underlying conditions (13). Unlike other bacterial infections that typically result from traumatic injuries, *C. septicum* infections are characterized by edema, extensive tissue damage, fluid-filled bullae, and thrombosis, which may lead to fatal outcomes. *C. septicum* produces several exotoxins, among which α-toxin is a critical virulence factor; however, no toxins are encoded in the bacterium's only identified plasmid (14, 15).

The *C. septicum* α-toxin is a β-pore-forming toxin (β-PFT) that is secreted as an inactive proprotein through the type II secretion system. Upon the removal of the C-terminal propeptide, the protein becomes active (16). This pore-forming exotoxin belongs to the aerolysin-like toxin family and targets glycosylphosphatidylinositol (GPI)-anchored proteins on the cell surface (17). Numerous GPI-anchored proteins, such as mesothelin, carcinoembryonic antigen, prostate-specific stem cell antigen, and urokinase plasminogen activator receptor, have been identified as tumor-associated antigens and are believed to promote tumorigenesis. Notably, α-toxin binds to the GPI glycan region (18).

Several studies have utilized *C. septicum* α-toxin to isolate and identify GPI-anchored proteins from human breast cancer tissues, cells, and serum. These investigations demonstrated increased binding of α-

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toxin to plasma proteins, suggesting elevated levels of GPI-anchored proteins in cancer patients, which is associated with increased GPI transamidase activity (18, 19).

The precise mechanism of action of *C. septicum* α-toxin remains unclear. However, due to the limited scientific research regarding the potential role of *C. septicum* α-toxin in cancer therapy, this study aimed to investigate its effects on cancer cell lines. In this study, the recombinant expression of α-toxin was achieved, followed by its purification to evaluate its anticancer properties on breast cancer cell lines.

Materials and methods

Ethical Approval

The Institutional Research and Ethics Committee of Kashan University of Medical Sciences approved all procedures performed in this study (No. IR.KAUMS.MEDNT.REC.1400.095).

Construction of the Recombinant Vector

E. coli BL21 (DE3) F^- *ompT gal dcm hsdSB* (rB^- mB^-) and the pET28a (+) plasmid were used as the host strain and vector, respectively. The coding sequence (CDS) of the *α-toxin* gene (Accession number: Q53482) was retrieved from the NCBI database. The *α-toxin* CDS was optimized using two online tools: OPTIMIZER (http://genomes.urv.es/OPTIMIZER) and the *E. coli* Unusual Codon Analyzer (http://faculty.ucr.edu/~mmaduro/codonusage/). The optimized sequence, consisting of 1,250 base pairs, was synthesized by GeneCust (Luxembourg) and subsequently cloned into the pET28a (+) vector.

Plasmid Extraction

A bacterial colony containing the pET28a (+) plasmid harboring the *α-toxin* gene was cultured overnight at 37°C and 200 rpm in Luria-Bertani (LB) broth supplemented with 50 µg/mL of kanamycin (Roche, Germany). Plasmid extraction was carried out using the Vivantis plasmid extraction kit (Malaysia, #GF-PL-050) following the manufacturer's protocol. The extracted plasmid was digested with *Nco*I and *Xho*I restriction enzymes (Thermo Scientific, USA), and the resulting DNA fragments were visualized on a 1% agarose gel.

Protein Expression

The genetically modified *E. coli* was inoculated into an LB medium containing 50 µg/mL of kanamycin and incubated overnight at 37 °C and 200 rpm. The overnight culture was then transferred to 50 mL of fresh LB medium. When the culture reached an optical density at 600 nm (OD_{600}) between 0.4 and 0.6, α-toxin expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; CinnaGen, Iran). Following induction, the incubation temperature was lowered to 30 °C, and the culture was incubated for 16 h. The bacterial cells were subsequently harvested, resuspended in lysis buffer, and subjected to sonication (five cycles of 30 sec each) to extract the total protein.

Protein Purification

The recombinant His-tagged α-toxin was purified using Ni-NTA resin (Ni-NTA Superflow, Cat. No. 30410, Qiagen) (20). A total protein sample (10 mL) obtained from 300 mL of recombinant bacterial culture was applied to the Ni-NTA column. The column was washed five times with a washing buffer (10 mM Tris base, 100 mM NaH_2PO_4, 8 M urea, pH 6.3) to eliminate unbound proteins. The recombinant protein was then eluted with two elution buffers at pH 5.9 and pH 4.5, both containing 10 mM Tris base, 100 mM

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NaH_2PO_4, and 8 M urea. To remove urea, the recombinant protein solution was dialyzed against phosphate-buffered saline (PBS) at 4 °C for 24 h. The protein concentration was determined using a bicinchoninic acid (BCA) assay kit (Parstous, Iran). SDS-PAGE analyzed eluted fractions to confirm the presence of purified α-toxin.

SDS-PAGE and Western Blot Analysis

Fractionated protein samples were subjected to SDS-PAGE using a 10% running gel and a 4% stacking gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane at 80 V for 90 min. The membrane was then blocked with 5% (w/v) skim milk in TBS for 1 hour at 25 °C. Following the blocking step, the membrane was incubated with a diluted anti-His horseradish peroxidase (HRP) antibody (1:1000, Sigma, USA) for 1 hour at room temperature. Visualization of the HRP-conjugated His-tagged protein was performed by adding the tetramethylbenzidine (TMB) substrate (21).

Cell Culture

The MCF-7 human breast cancer cell line (NCBI Code: C135), known for its spindle-shaped morphology, was obtained from the Cell Bank of the Pasteur Institute in Tehran, Iran, in cryopreserved vials. Cells were seeded into T75 cell culture flasks at a density of 1×10^6 cells/flask and maintained at 37 °C in a humidified incubator with 5% CO_2. The cells were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 20% fetal calf serum (FCS), 0.01 mg/mL insulin, and 1% penicillin/streptomycin (PenStrep, Gibco 15140).

Cells were separated from the flask using a 0.025% trypsin-EDTA solution, and cell counting was performed using a hemocytometer. In all assays, the viability of cells, as determined by trypan blue exclusion, exceeded 95%. The medium was replaced twice weekly, and cells were sub-cultured weekly at a 1:3 ratio (11). An alternative medium, DMEM supplemented with antibiotics/antimycotics and 10% fetal bovine serum (FBS), was also considered (12).

Evaluation of anticancer activity

After thawing, the cells were washed and transferred to T75 flasks containing RPMI 1640 medium supplemented with 20% fetal calf serum (FCS), 100 U/mL penicillin, and 100 µg/mL streptomycin. The flasks were then incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 5–6 days. Cells were separated using trypsin-EDTA and washed with RPMI 1640 medium. Cell counting was performed using a hemocytometer.

A total of 2×10^4 cells were seeded into each well of a microplate containing RPMI 1640 medium and incubated at 37 °C with 5% CO_2 for 24, 48, and 72 h. After each incubation period, the supernatant was removed, and the cells were treated with varying concentrations of α-toxin (0.5, 5, and 10 µg/mL). Following α-toxin treatment, the cells were incubated under humidified conditions at 37°C with 5% CO_2 for 1 hour. Each treatment was performed in triplicate. Untreated cells served as the negative control. The half-maximal cytotoxic concentration (CC_{50}) of α-toxin on the MCF-7 cell line was determined using the MTT assay and analyzed with GraphPad Prism 7 software.

***In vivo* experiments**

Forty female Balb/c mice, aged 4–6 weeks, were obtained from the Royan Institute, Isfahan, Iran. Breast cancer was induced in the mice by intradermally inoculating each animal with 1×10^6 MCF-7 cells at the base

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of the tail. After three weeks, tumour nodules developed, and the mice were divided into four groups (n = 10 per group) based on the amount of α-toxin administered. The groups were as follows: 1) 0.5 µg/kg α-toxin, 2) 5 µg/kg α-toxin, 3) 10 mg/kg α-toxin, and 4) an untreated group serving as the negative control.

According to the treatment protocol, α-toxin was injected intradermally daily for three weeks. Tumour nodule sizes were measured weekly, both before and after treatment, using a Vernier calliper to assess therapeutic efficacy.

Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM). Statistical analyses were conducted using one-way analysis of variance (ANOVA) and Student's t-test, performed with SPSS software version 18. A p-value of less than 0.05 was considered statistically significant.

Results

Digestion Confirmation

The accuracy of the pET28a-*α-toxin* plasmid was verified through *Nco*I/*Xho*I restriction enzymes digestion, which yielded two distinct fragments of 5,234 bp and 1,250 bp on agarose gel (Fig.1A).

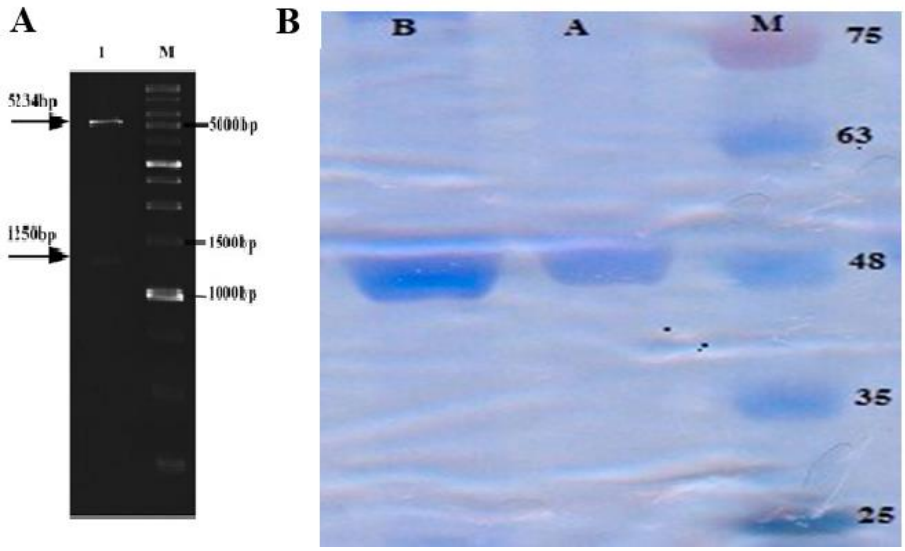


Fig. 1. A) Enzymatic digestion of pET28a(+) containing the *α-toxin* gene. **Lane 1**: digested vector with *Nco*I/*Xho*I restriction enzymes. Arrows indicate the desired 1,250 bp band corresponding to the *α-toxin* CDS and the 5,234 bp band representing the plasmid backbone. **M:** DNA Ladder 1 kb (Thermo Fisher). **B)** SDS- PAGE analysis of the purified recombinant α-toxin protein using Ni-NTA resin. **M:** Pre-stained protein marker (Sinaclon, Iran) (10–170 kDa). Lanes **A** and **B** show the eluted fractions 2 and 4 of the recombinant protein from *E. coli* BL21 (DE3) transformed with pET28a (+)-α-toxin-6×His tags.

Expression and Purification of Recombinant α-Toxin

The recombinant vector was successfully transformed into *E. coli* for α-toxin production. SDS-PAGE analysis revealed the presence of a 48-kDa band, corresponding to the α-toxin fused with 6×His tags (Fig. 1B). To validate the identity of the α-toxin-6×His fusion further, Western blotting was performed using an antibody-specific for the 6×His tag (Figure 2). The protein concentration was estimated to be approximately 1 mg/mL.

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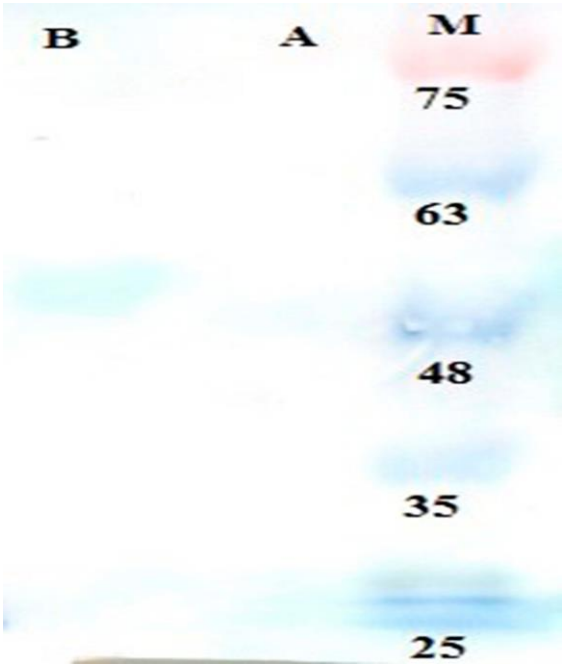


Fig. 2. Western blot analysis of purified recombinant α-toxin-6×His-tagged protein. **M)** Pre-stained protein ladder (Sinaclon, Iran). **Lane A:** Negative control (*E. coli* BL21 (DE3)). **Lane B)** Purified recombinant protein from *E. coli* BL21 (DE3) transformed with pET28a (+)-α-toxin-6×His tags, showing a single band at 48 kDa.

Evaluation of Anticancer Effect of Recombinant α-Toxin

The half-maximal cytotoxic concentration (CC_{50}) of α-toxin on MCF-7 cells was determined to be 407.3 ± 2.392 µg/mL. Importantly, the α-toxin showed no cytotoxic effects at concentrations equal to or below 0.003 µg/mL. After assessing the effects of various concentrations (0.5–10 µg/mL) of α-toxin on MCF-7 cells compared to the negative control, the IC_{50} values were found to be 407.3 ± 2.392 µg/mL, 287.3 ± 5.411 µg/mL, and 258.1 ± 4.671 µg/mL at 24, 48, and 72 h, respectively (Table 1; Figs. 3, 4). The concentration required to achieve a 50% reduction in cell viability was 407.3 µg/mL ± 2.392, indicating a strong anticancer effect in MCF-7 cells compared to the negative control. These findings demonstrate that α-toxin (IC_{50} = 407.3 µg/mL ± 2.392) exerted a significant anticancer effect on MCF-7 cells (Figure 5).

***In vivo* Assessment**

Nodule sizes were measured on day 0 and throughout the 3-week treatment period. The results of the ANOVA test indicated significant effects of both time ($p < 0.001$) and treatment groups ($p = 0.57$) on nodule size. Although changes in ulcer size were observed over time, they varied across the four groups. A notable reduction in nodule size was observed in the group treated with 10 µg/kg of α-toxin compared to the group receiving 0.5 µg/kg of α-toxin, where nodule size remained the largest ($p < 0.001$). In contrast, the nodule size in the negative control group increased significantly over time compared to the three treatment groups (Figure 6). The One-Way ANOVA test showed no significant differences in mean nodule diameters among the four groups before treatment ($p = 0.57$). However, significant differences were observed at weeks 1, 2, and 3 post-treatment (Figure 7).

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Table 1. IC_{50} statistical analysis at different time points.

Descriptive statistics	24 h	48 h	72 h
Mean of IC50 (µg/mL)	413.900	300.500	269.100
SD	2.392	5.411	4.671
Lower 95% CI	407.3	287.2	258.1
Upper 95% CI	419.2	314.1	281.3

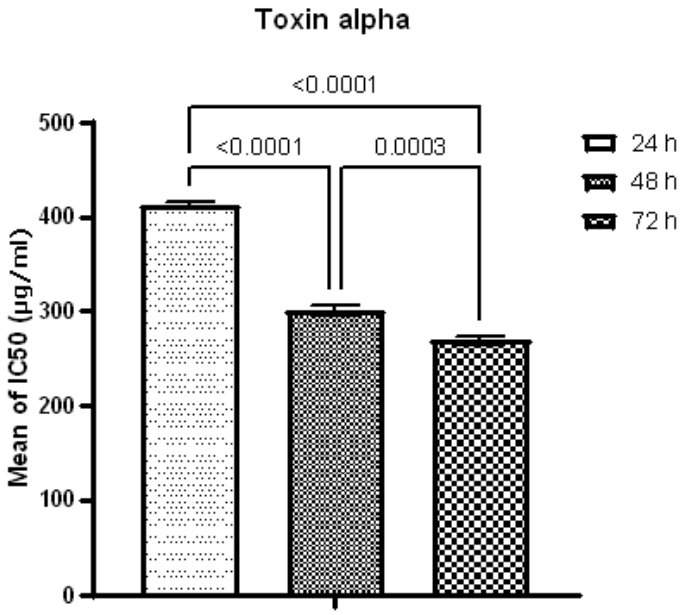


Fig. 3. Comparison of the average IC_{50} values at different time points. The curve demonstrates that α-toxin exerted a more significant anticancer effect on MCF-7 cells, with an IC_{50} of 407.3 µg/mL ± 2.392 at 24 h.

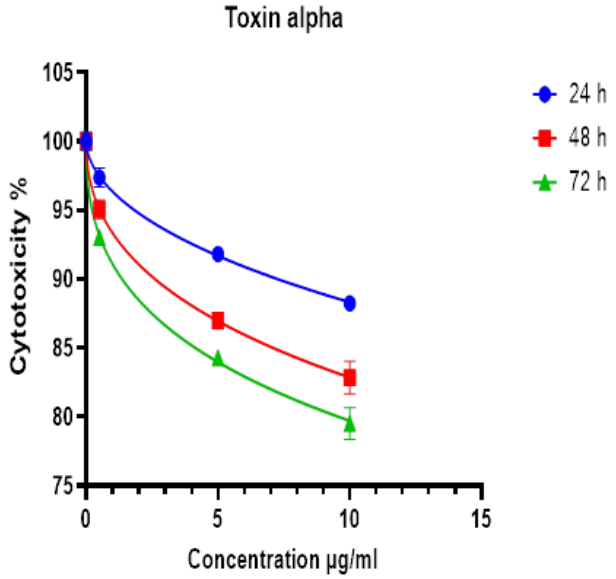


Fig. 4. Comparison of cytotoxicity of α-toxin at different time points.

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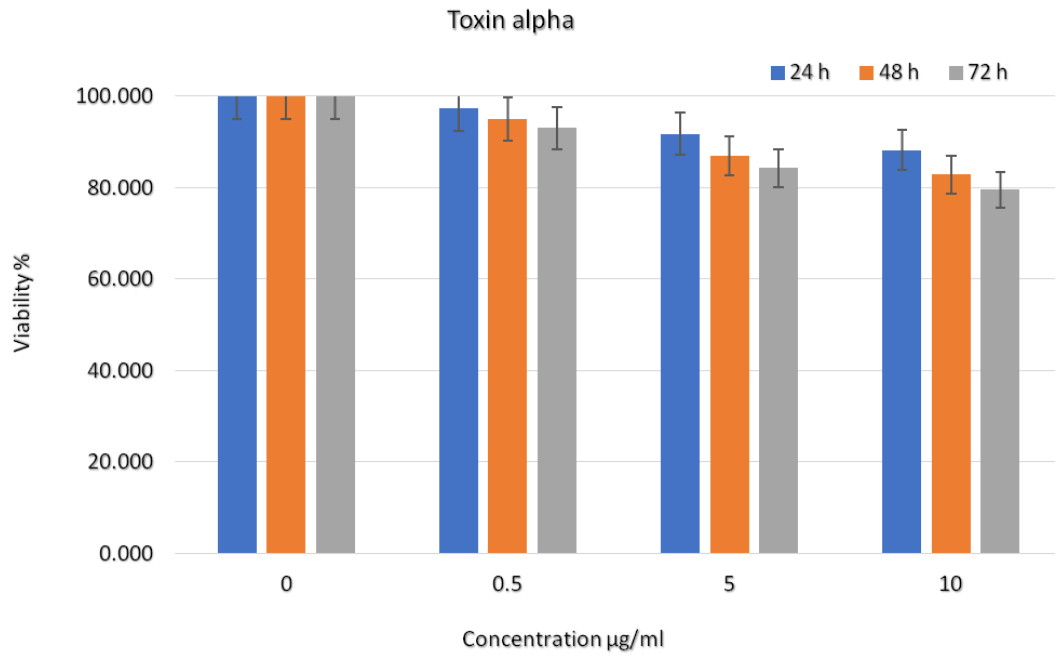


Fig. 5. Comparison of MCF-7 cell survival rates after treatment with different concentrations of α-toxin over time. The protein exhibited no cytotoxic effect at concentrations below 0.003 µg/mL, while the highest cytotoxic effect was observed at a concentration of 10□µg/mL.

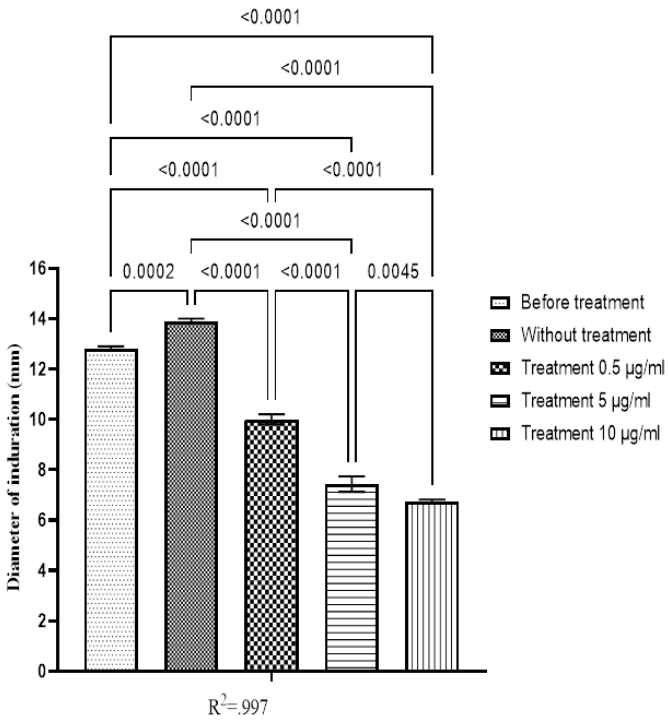


Fig. 6. Comparison of MCF-7 nodule diameters in rats before and after treatment with different concentrations of α-toxin. A significant reduction in nodule size was observed in the group treated with 10 µg/kg of α-toxin compared to the other treated groups. In contrast, the nodule size increased over time in the negative control group.

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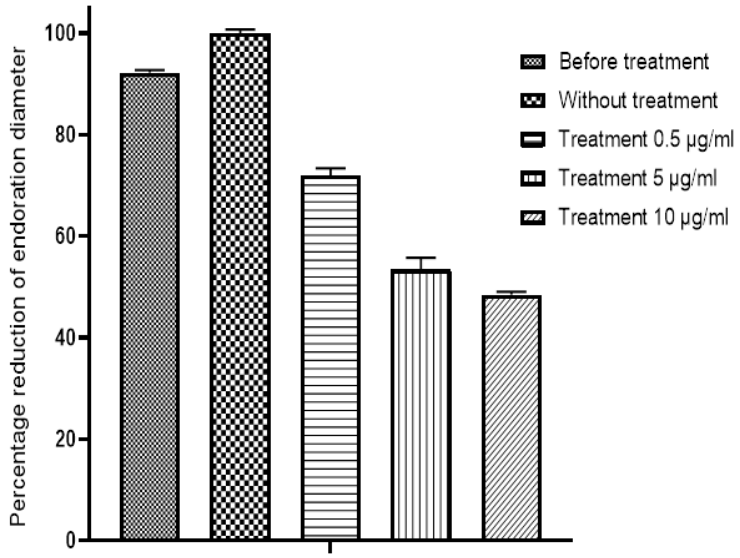


Fig. 7. Comparison of the percentage reduction in MCF-7 nodule diameters in rats before and after treatment with different concentrations of α-toxin. The group treated with 10 µg/kg of α-toxin exhibited the highest percentage reduction in nodule diameter, while the negative control group showed the lowest reduction.

Discussion

Despite initial positive responses in patients, a definitive cure for cancer has yet to be discovered (22, 23). In addition to synthetic and herbal medications, bacterial metabolites, including toxins, have shown potential as therapeutic agents against various types of cancer, either alone or in combination with conventional treatments (24). In recent years, anaerobic *Clostridium* species have been investigated for their ability to target treatment-resistant hypoxic tumors (25-27). The *C. septicum*- α-toxin is capable of recognizing GPI-anchors on eukaryotic cells and plays a key role in muscle necrosis (17) by inducing small ion-selective permeability in cell membranes, leading to potassium ion (K^+) efflux (19). This alteration in membrane permeability occurs through the binding of α-toxin to both gp63 and GPIs, ultimately resulting in hemolysis and cellular necrosis (28). The targeted use of recombinant bacterial toxins offers promising potential in advancing therapeutic strategies, particularly for diseases such as breast cancer (29).

In this study, a pET28a (+) plasmid containing an optimized synthetic coding sequence (CDS) of *α-toxin* was constructed and subsequently transformed into *E. coli* BL21 (DE3) cells. Codon optimization was employed as an effective strategy to enhance production yields and facilitate the efficient expression of α-toxin in *E. coli* BL21. The expression level of the recombinant α-toxin protein was approximately 1 mg/mL, attributed to both codon optimization and optimized culture conditions. Due to the lethality associated with high-level expression of recombinant α-toxin in *E. coli*, induction with IPTG was limited to 2 h. The molecular weight of the engineered protein was estimated to be approximately 48 kDa, as confirmed by SDS-PAGE and Western blot analyses.

In addition to its direct cytotoxic effects, the potential of α-toxin in cancer immunotherapy has garnered increasing interest. Schoepe *et al.* study investigated the immunogenic properties of recombinant α-toxin and demonstrated its ability to elicit protective immune responses in a mouse model. These findings suggest that

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α-toxin could be considered for the development of cancer vaccines or as an adjuvant in immunotherapeutic strategies (30).

In 2007, Inoue *et al.* successfully expressed the *α-toxin* gene from *C. perfringens* in *E. coli*. Similarly, Zhang *et al.* conducted a parallel study in which they cloned and expressed the *α-toxin* gene from *C. septicum* using the pQE30 expression vector in *E. coli*. In their study, they produced a toxoid vaccine by incorporating 0.3% formaldehyde into the expressed α-toxin. Additionally, Zhang *et al*. demonstrated that the recombinant strain M15 (pQE30-αα) showed promise as a candidate for protective immunization against *C. septicum* infections (31). These findings provide valuable insights into the expression and potential immunogenic applications of *α-toxin* genes from different *Clostridium* species.

Various researchers have explored the lethal effects of α-toxin on cells and its interactions with infectious agents. For instance, Gordon *et al*. documented the cytotoxic impact of α-toxin on Chinese hamster ovary (CHO) cells transfected with GPI-anchored folate receptors. Their research highlighted the specific cellular responses induced by α-toxin, offering valuable insights into its cytotoxic effects and potential applications in the study of cellular processes (32). Additionally, Biazar *et al*. demonstrated the cytocidal activity of *Bacillus thuringiensis* crystal protein on 4T1 breast cancer cells in a murine model. Their findings revealed that parasporin, identified as a cytolysin protein, interacted specifically with the cell surface, ultimately inducing cell death in 4T1 breast cancer cells (33).

In 2004, Ishitoya *et al.* investigated the effects of different concentrations of verotoxin type 1 on liver carcinoma, prostate, and testicular tumors. Their study demonstrated significant inhibition of tumor cell growth upon exposure to verotoxin type 1, suggesting potential anti-tumor effects of this toxin on various types of cancer cells (34).

Comparative analyses with conventional anticancer agents indicate that, unlike many chemotherapeutic drugs, which lack specificity and damage healthy cells (28), α-toxin selectively targets cancer cells via specific receptors, offering a novel therapeutic approach (35). This targeted mechanism is further supported by research conducted by Salhia *et al.* (2002), which demonstrated the use of verotoxin type 1 in the treatment of intracranial tumors and its role in inhibiting tumor vascularization (36).

Furthermore, Takagishi demonstrated that α-toxin stimulates GM1a clustering and TrkA phosphorylation in the host cell membrane. His study identified the specific site on the cell surface where α-toxin binds, triggering a cascade of events that lead to programmed cell death. This targeted cytotoxicity highlights the potential of α-toxin in cancer therapy (37).

Moreover, Namdar *et al*. demonstrated the collaborative efficacy of recombinant α-toxin protein from *C. septicum* in combination with glucantime for the eradication of *Leishmania* parasites (38).

In agreement with previous findings, the assessment of α-toxin IC_{50} in the current study revealed a significant difference in the mean count of MCF-7 cells among the groups treated with α-toxin compared to the negative control group. The therapeutic efficacy of α-toxin on MCF-7 cells in mice demonstrated a substantial impact.

Moreover, the average diameter of nodules in the groups treated with α-toxin decreased significantly compared to the control group, indicating a pronounced effect of α-toxin on MCF-7 cells. Due to its biological

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properties, particularly its ability to form pores in the cell membrane, α-toxin may enhance drug delivery and exhibit synergistic effects with conventional cancer therapies.

In recent decades, bacterial toxins have been extensively studied for their potential efficacy against cancer cells (25), highlighting the promise of utilizing bacteria or their metabolites as effective cancer treatments. The findings of this research are expected to contribute valuable insights into future cancer therapy strategies.

While the anticancer potential of α-toxin is promising, challenges such as systemic toxicity and immune responses must be addressed before progressing to clinical trials. In the current study, *C. septicum*-α-toxin demonstrated potential as a promising agent in anticancer therapies. The toxin indicated significant anticancer effects on MCF-7 cells *in vitro*. In a breast cancer mouse model, it caused a significant reduction in nodule size in vivo compared to the control group. The specific mechanism of α-toxin, which represented cytotoxic effects on cancer cells, as well as its potential applications in immunotherapy, make it a candidate for further investigation. Future research should focus on optimizing dosage, exploring alternative delivery methods, and engineering *α-toxin* for enhanced specificity.

Acknowledgments

This study was conducted in the Department of Microbiology and Immunology, School of Medicine, Kashan University of Medical Sciences, Kashan, Iran. The results presented in this article are part of a PhD thesis supervised by the last author.

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