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ORIGINAL ARTICLE

Cell death analysis of recombinant mature epsilon toxin on the kidney cell line

Roza Chehreara[1], Shohreh Zare Karizi[2], Hamideh Mahmoodzadeh Hosseini[3], Seyed Ali Mirhosseini[3*], Mohammad Shafiei[3], Jafar Amani[3], Rouhollah Kazemi[4]

[1]Department of Genetics, Faculty of Basic Sciences, Islamic Azad University, Research Branch, Tehran, Iran
[2]Department of Genetics and Biotechnology, School of Biological Science, Varamin-Pishva, Branch Islamic Azad University, Varamin, Iran
[3]Applied Microbiology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran
[4]Department of Plant Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran

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ABSTRACT

Background and Objectives: Epsilon toxin is the third hazardous bacterial toxin causing ABS enterotoxaemia in domestic animal. In addition, epsilon toxin is known as a biological warfare agent. The aim of this study was to produce the recombinant mature epsilon toxin to evaluate cell death impact on the kidney cell line.
Materials and Methods: For this purpose, the sequence of mature epsilon toxin (46-328 aa) in pET28a was cloned and expressed in *Escherichia coli* BL21 (DE3) and purified by nickel-nitrilotriacetic acid (Ni-NTA) column and confirmed by western blot analysis using HRP conjugated anti-His antibody. Then, to assess the anti-proliferative effects of different concentrations of recombinant epsilon toxin, the MTT assay was done on the HEK293 cell line. The annexin V/PI staining was done to investigate the apoptotic and necrotic cell populations after exposure to epsilon toxin.
Results: Induction by 1 mM IPTG for 4 h at 37°C was an optimized condition for expressing mature epsilon toxin in *E. coli* strain BL21 (DE3). Electrophoresis on SDS-PAGE 12% gel showed the desired band approximately at 38 KDa. Our results showed that recombinant epsilon toxin is mainly expressed as an inclusion body. Furthermore, 100, 150, and 200 µg/mL of mature epsilon toxin are significantly reduced the cell viability ($P \leq 0.05$). The considerable increase of necrotic cell percentage was shown after exposing to 100, 150, and 200 µg/mL of mature epsilon toxin ($P \leq 0.05$).
Conclusion: The recombinant mature epsilon toxin had cytotoxic effects and could induce necrosis.

Keywords: Cell death; *Clostridium perfringens*; Epsilon toxin; Necrosis; Recombinant expression

INTRODUCTION

Clostridium perfringens is the anaerobic, Gram-positive bacillus bacterium that is able to produce spores. It is a non-motile bacterium (1, 2). *C. perfringens* was categorized into seven groups named A to G following the type of toxin. Epsilon toxin (ε-toxin, ETX) was produced by both *C. perfringens* type B and D and is the third hazardous toxin after tetanus and botulinum toxins that it is known as a biological warfare agent (3). Lethal Dose 50 (LD_{50}) of epsilon toxin is approximately 70 ng/Kg

*Corresponding author: Seyed Ali Mirhosseini, Ph.D, Applied Microbiology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran. Tel: +98-21-82482568 Fax: +98-21-88068924 Email: ali.mirh@gmail.com

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body weight in the mouse.

Epsilon toxin is able to create enteric diseases in the cattle such as sheep and goats (4). After infection with *C. perfringens* type B and D, the high quantity of epsilon toxin is secreted into the intestinal lumen of infected animals and absorbed by mucosa. After that, increased blood pressure, vascular permeability, and intense vascular destructions occur. The main target organs are the brain, heart, lung and kidney and lesions and necrosis are the common pathologic manifestations occurring after epsilon toxin intoxication (5-7).

Epsilon toxin has two forms, the mature and immature forms. The immature form is longer and has 23 more amino acids at C- terminal. It is known as protoxin without biological activity. The second form, mature toxin, creates after cleavage of the protoxin by proteases, and it is an active form of epsilon toxin (8).

Epsilon toxin consists of three parts including domain I as the receptor-binding domain (9), domain II for the membrane insertion (4), and domain III for monomer interactions and oligomerization (5). Several researchers studied the cytotoxicity effect of epsilon toxin on the different cell lines. MDCK, as Madin Darby Canine Kidney cell line, is the most frequently applied to investigate the cellular effects of epsilon toxin. According to works on MDCK cells, it was observed that epsilon toxin destroyed the cell membrane and raised the cell permeability via creating a pore in the cell membrane that it caused to disrupt the ionic balance inside and outside the cells. Increased cellular concentration of Na^+, Cl^-, and $Ca2^+$ and reducing cellular concentration of K^+ happened after epsilon toxin exposure (10, 11). In addition, epsilon toxin leads to the malfunction of the cytoskeleton (12). However, a few studies were done on the human embryonic kidney (HEK293) and G402 cells, human erythrocyte and Human Kidney Adenocarcinoma (ACHN) (13-15). HEK 293 cells and their derived cell lines are widely used in research and biotechnology. Also, embryonic stem cells and adult human cell lines are refractory to the oncogenic potential of cancer patient sera. HEK293 are immortalized cells (16) prone to malignant transformation following transfer of oncogenes (17). Therefore, obtaining information about the effect of toxin on this cell line, in addition to clarifying the function of the toxin, can also help to study and kill the toxicity of toxin cancer cells. Therefore, the purpose of the current study was to produce recombinant mature epsilon toxin and assess its effects on the cell death of HEK293 cell lines.

MATERIALS AND METHODS

Design the epsilon toxin construct. The whole sequence of *etxB* gene of *C. perfringens* (Q02307) was extracted from UniProtKB. ETXB contains three parts; signal peptide from 1 to 32 aa, propeptide from 33 to 45 aa, and toxin from 46 to 328 aa. In this study, to express the active epsilon toxin, the toxin part (46-328 aa) was selected. The start codon was added to the 5' end of the toxin gene. In addition, the restriction site for *Eco*RI and *Hind*III was added to the 5' and 3' end of the construct, respectively (Fig. 1).

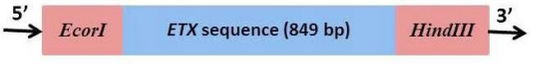

Fig. 1. Schematic image of *etxB* construct

The pET28a containing our construct (*etx*B- pET28a) was synthesized by Biomatic Company, Canada. The *etx*B- pET28a expression vector was transformed into *E. coli* BL21 (DE3) (Gift from National Genetic Institute, Tehran, Iran) competent cells via heat shock method and cultured on Luria Bertani agar (LB, Quelab, Canada) with 50 µg/mL kanamycin (Merck, Germany) for 16h at 37°C. The colonies were transferred into 5 mL LB broth (Quelab, Canada) containing 50 µg/mL kanamycin overnight at 37°C. To confirm the presence of desired gene, the plasmid was isolated using GeNet Bio kit, Korea, according to the manufacturer's guideline, and the gene was digested with *Eco*RI and *Hind*III (Fermentase, Canada) and run on 1.5% agarose gel (Sinaclon, Iran). Finally, the gene was analyzed via DNA sequencing. Then, to optimize the expression of ETX B protein, the transformed colony was added to 5 mL LB broth containing 50 µg/mL kanamycin and incubated at 37°C and 150 rpm till the optical density of culture medium at 600 nm reached 0.7. Next, to induce the protein expression, 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, USA) was added to culture medium and after 2, 4, and 16 h, 1 mL of culture medium was collected to investigate the expression level using SDS-PAGE electrophoresis.

Recombinant protein purification and confirmation. To purify the recombinant protein, nickel-nitrilotriacetic acid (Ni-NTA) column (Sigma-Aldrich, USA) was applied. Briefly, transformed *E. coli* BL21 (DE3) was cultured in LB broth containing 50 µg/mL kanamycin and 1 mM IPTG for 4 h at 37°C and 150 rpm. Then, the medium was centrifuged at 3500 rpm for 10 min to isolate the bacterial cell pellet. To break the bacterial cells, 1 mL lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, at pH 8) and 1 mg/mL lysozyme (Sinaclon, Iran) were added to the cell pellet under native condition and shaken for 30 min. After centrifugation at 12000 rpm at 4°C for 30 min, clear supernatant was transferred to Ni-NTA column. The column was washed with wash buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 30 mM Imidazole at pH 8 (Sigma-Aldrich, USA). After that, the elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM Imidazole at pH 8) was added to the column and collected the elutes. The elutes were analyzed by electrophoresis on 12% SDS-PAGE. The quantity of purified protein was measured using Bradford assay.

Finally, to confirm the purified protein, western blot was performed on nitrocellulose membrane using mouse anti-His antibody conjugated with HRP (horseradish peroxidase) (Sigma, USA). The 3, 3′-Diaminobenzidine solution (Sigma-Aldrich, USA) was applied as a substrate solution.

Cytotoxic effect analysis of epsilon toxin. To assess the cytotoxic effects of recombinant epsilon toxin, MTT (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide) assay was carried out. HEK293 cells (ATCC # CRL-1573), the human embryonic kidney cells, were obtained from Pasteur institute cell bank (Tehran, Iran). HEK293 cells were cultured in RPMI1640 (Gibco, USA) containing 1% penicillin/streptomycin (Gibco, USA) and 10% fetal bovine serum (FBS) (Gibco, USA) at 37°C and 5% CO_2. After reaching 80% confluency, HEK293 cells were detached using Trypsin/EDTA (Gibco, USA) and 8000 cells were harvested in each well of 96-well plate containing RPMI1640 containing 1% penicillin/streptomycin and 10% FBS. After overnight incubation at 37°C and 5% CO_2, the cells treated with 0.97, 1.95, 3.90, 7.80, 15.62, 31.25, 62.50, 125, 250, 325, 500, 1000, 2000, 2500, 3000 and 4000 µg/mL epsilon toxin for 24h at 37°C and 5% CO_2. After that, the medium was exchanged with the 200 µL RPMI1640 containing 20 µL MTT solution (5 mg/mL) (Sigma-Aldrich, USA) and incubated for 4 h at 37°C and 5% CO_2. Then, the medium was replaced with 100 µL dimethyl sulfoxide (Sigma-Aldrich, USA), and the optical density of each well was recorded at 570 nm using a microtiter plate reader (Biorad, USA). Phosphate buffer saline (PBS) was tested as the negative control. All tests were triplicated and analyzed three times.

Cell death analysis. To survey the effect of epsilon toxin on the cell death of HEK293 cells, the annexin V/PI staining was done. Briefly, 1×10^6 cells were added to each well of 24-well plate containing 1 mL culture medium. After overnight incubation at 37°C and 5% CO_2, the cells were treated with 100, 150, and 200 µg/mL epsilon toxin for 24 h at 37°C and 5% CO_2. Then, cells were detached with trypsin/EDTA and washed with PBS. 1 mL of Annexin V-binding buffer and 5 µL Annexin V-coupled FITC and propidium iodide (BD, USA) was added to cells. After gently mixing the cells at room temperature for 15 min, the percentage of stained cells was measured using the flow cytometry assay (FACSCalibure instrument from BD Biosciences). The data recording from the flow cytometry assay was analyzed by FlowJo software.

Statistical analysis. Differences between tested groups were analyzed using one-way ANOVA by the SPSS software version 15 (SPSS Inc., Chicago, IL., Iran). P value <0.05 was reported statistically significant.

RESULTS

To confirm the pET28a containing *etx*B gene, double digestion by was performed. As shown in Fig. 2, two bands with the different size were created after digestions that are related to plasmid and *etx*B gene.

Expression of recombinant epsilon toxin. Findings from induction of two transformed *E. coli* strain BL21 (DE3) by 1 mM IPTG after 2, 4, and 16 h were illustrated in Fig. 3. Induction by 1 mM IPTG for 4 h at 37°C was an optimized condition for expression of mature epsilon toxin in *E. coli* strain BL21 (DE3). Electrophoresis on SDS-PAGE 12% gel showed the desired band approximately at 38 KDa. Our results showed that recombinant epsilon toxin is mainly expressed as an inclusion body.

Protein purification and isolation. The expressed

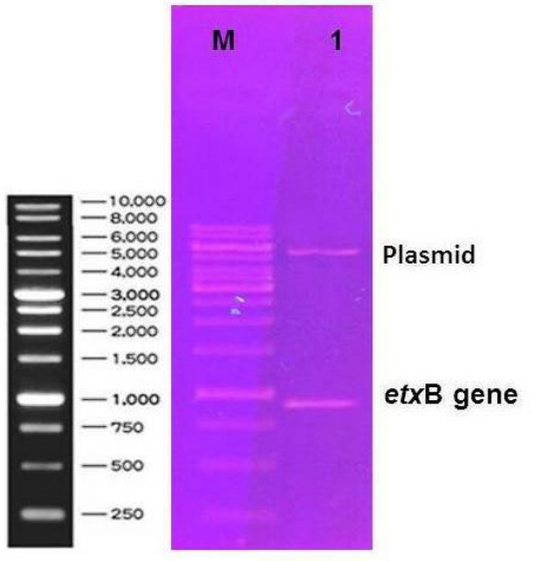

Fig. 2. Double digest of pET28a containing *etx*B construct using *Ecor*I and *Hind*III. Two bands in lane 1 relate to plasmid and *etx*B gene. M indicates DNA ladder and lane 1 indicates digestion products.

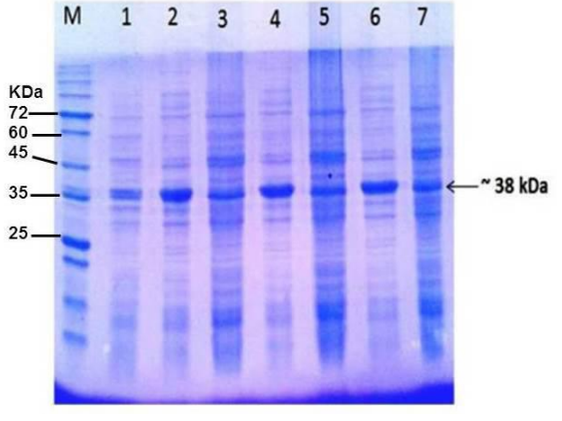

Fig. 3. Protein expression of *etx*B on the 12% SDS-PAGE. Lane 1: uninduced sample; lane 2, 3 and 4 indicate induction clone 1 by 1 mM IPTG after 2, 4, and 16 h, respectively; lane 5, 6 and 7 indicate induction clone 2 by 1 mM IPTG after 2, 4, and 16 h, respectively; M indicates protein marker.

epsilon toxin was purified by Ni-NTA affinity column that was bound to its Histidine tag. Fig. 4A showed data from protein purification in the denature condition with 8 M urea. In addition, Fig. 4B presented the findings from protein purification via the native condition with 250 mM Imidazole. Fig. 4C illustrated the western blot analysis with the mouse anti-his antibody that confirmed the presence of approximately 38 KDa band related to recombinant epsilon toxin in the purified sample. In this study, the protein was expressed as both soluble and insoluble forms. The protein concentration of soluble and insoluble forms was 60 and 772 µg, respectively.

Cell viability test. The effect of mature epsilon toxin on the proliferation of HEK293 cells was investigated using MTT assay. Our data revealed the 100, 150 and 200 µg/mL of mature epsilon toxin are considerably able to attenuate the cell viability percentage (80, 68, and 60%, respectively) in comparison with the negative control (100%) ($P \leq 0.05$) (Fig. 5). Furthermore, mature epsilon toxin had no cytotoxic impact from 5 to 80 µg/mL.

Cell death analysis. To investigate the pattern of cell death in the HEK293 cells after exposing to mature epsilon toxin, annexin V/PI staining was performed. We observed the significant increase of necrotic and late-stage apoptotic cell percentage after exposing to 100, 150, and 200 µg/mL of mature epsilon toxin (17.7, 23, and 19.8%, respectively) compared with negative control (3.67%) ($P \leq 0.05$) (Fig. 6). The percentage of stained cells in the apoptotic area (Q2+Q3) had no significant differences compared with the control group.

DISCUSSION

Previous findings indicated that epsilon toxin is the pore-forming toxin, and its activity depends on the cell types and cell sensitivity. Previous reports showed that the cell death mechanism of various pore-forming toxins is programmed necrosis. Programmed necrosis occurs due to ischemia, cell damage, and cytotoxicity (18). There is a primary stage, named the pre-pore stage, that occurs during the first 60 minutes after exposure to epsilon toxin and is sensitive to temperature (19). As the majority of previous studies have reported, the different cell types have different sensitivities to epsilon toxin, and MDCK cells are the most sensitive type because of higher receptors on their surface (20). It is observed that epsilon toxin binds to unspecific receptors on the cell surface at 37ºC and oligomerizes to form a precursor pore complex (21). In this study, the protein was expressed as both soluble and insoluble forms. The protein concentration of soluble and insoluble forms was 60 and 772 µg, respectively. Also, the expressed

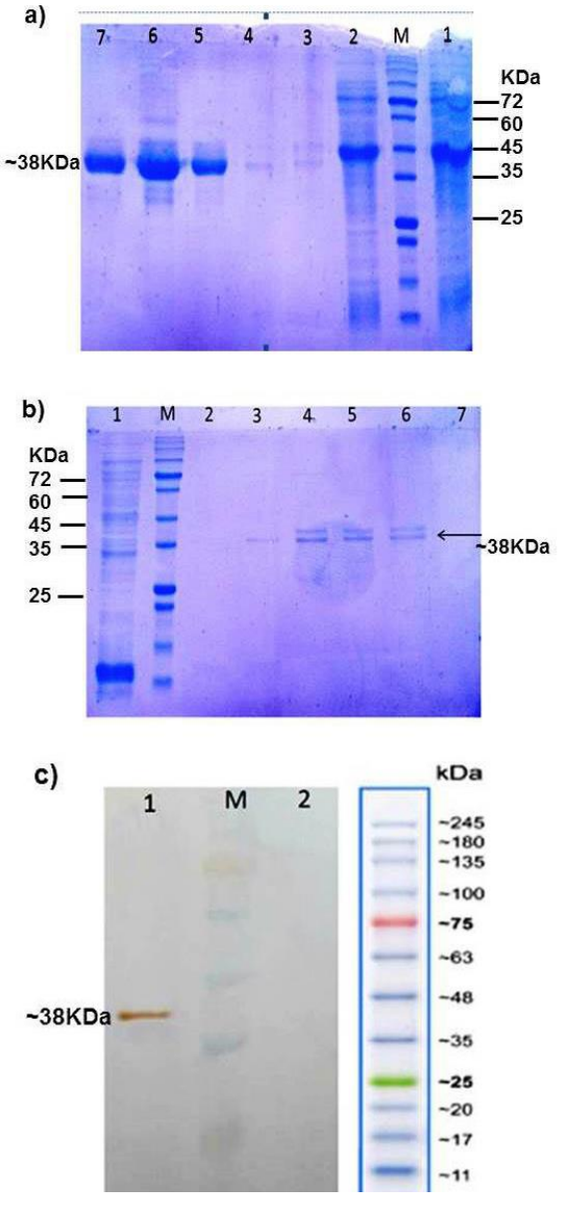

Fig. 4. Data from protein purification. a) Protein purification in the denature condition with 8 M urea on the 12% SDS-PAGE. Lane 1and lane 2: wash with 8 M urea, lane 3, 4, 5 and 6: elution 1, 2, 3 and 4, respectively, lane 7: wash with MES buffer and M: protein marker. b) Protein purification via the native condition with 250 mM Imidazole on the 12% SDS-PAGE. Lane 1and lane 2: wash with 250 mM Imidazole, lane 3, 4, 5 and 6: elution 1, 2, 3 and 4, respectively, lane 7: wash with MES buffer (dH2O, 2-(N-morpholino) ethanesulonic acid, NaOH) and M: Protein markers. c) Confirmation of the purified protein by Western blot analysis using anti-his antibody. M, lane 1 and lane 2 indicate Protein markers, purified epsilon toxin and negative control, respectively.

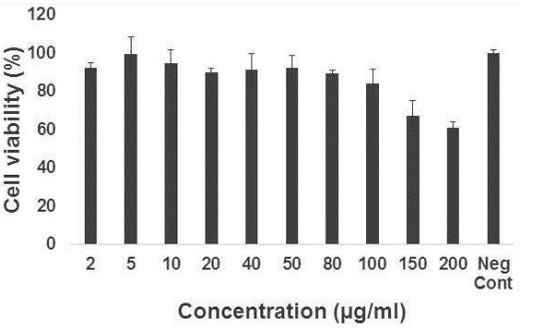

Fig. 5. The cell viability percentage after exposure to different concentrations of epsilon toxin

epsilon toxin was purified by Ni-NTA affinity column that was bound to its Histidine tag. Purifying a protein under native conditions (see example below) is the most efficient way to preserve its biological activity, but requires that the protein is soluble. Advantages include: Eliminating the renaturation step at the end of the purification, saving time, and preventing significant loss of activity. Additionally, we assessed the HEK293 cell line and observed that its sensitivity to epsilon toxin is lower than MDCK cells. The cell proliferation inhibitory concentration of epsilon toxin was 100 µg/mL for HEK293 cells that is 5 times higher than for MDCK cells (20 µg/ml) (22).

In the study conducted by Ferrarezi and co-workers, MDCK cell was treated with 20 µg/mL of both mature and immature epsilon toxins and the toxin effects were evaluated using acoustic flow cytometry. They observed that during 1 to 5 hours exposing to mature epsilon toxin, the cell viability percentage attenuated. In addition, cell membrane vacuolation and mitochondrial membrane depolarization happened after an hour, but after 5 hours, vacuolation decreased owing to cell damage. On the other hand, BAX/BCL-2 expression ratio as the apoptotic index increased over time, and it was higher after 5 hours exposing (22).

Moreover, Borrmann et al. reported the reducing effect of epsilon toxin on the cell proliferation of MDCK cells. They found an increase in cell volume and the number of cells in the S phase but the inability of cells for dividing and differentiation after 24 and 48 hours of exposure to epsilon toxin (20). In another study, Chassin et al. reported the necrotic mechanism of epsilon toxin via cellular ATP depletion, increased mitochondrial membrane permeabi-

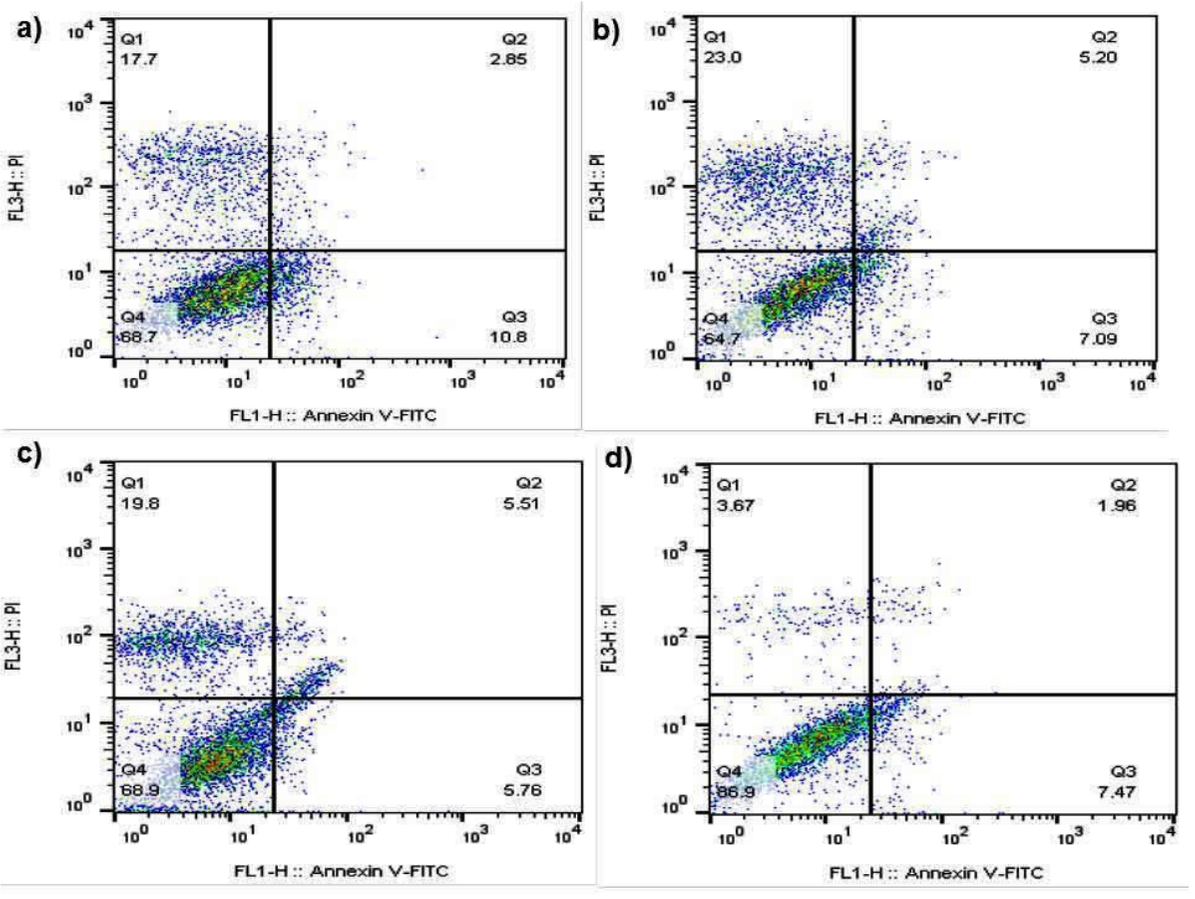

Fig. 6. Data from Annexin V/PI staining of HEK293 cells treated with 100 (a), 150 (b) and 200 µg/mL (c) of mature epsilon toxin and PBS as the negative control (d)

lization, and decreased nucleus size and chromatin condensation without DNA fragmentation (23).

Similar to most previous studies, our findings showed the necrotic effects of epsilon toxin on the HEK293 cell lines after 24 hours. Increase necrotic cells after epsilon toxin was shown using annexin V/PI staining. The high effective concentration of epsilon toxin and slight but significant increase of necrotic cell numbers could be due to the lower sensitivity of HEK293 cells to epsilon toxin.

In conclusion, the recombinant mature epsilon toxin has similar effects to native epsilon toxin and is able to inhibit proliferation and cell viability and induce necrosis on the HEK293 cells.

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