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Production and secretion of TNF related apoptosis inducing ligand (TRAIL/Apo2L) in the *Escherichia coli* periplasm using PhoA signal peptide

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The ability of TNF related apoptosis inducing ligand/Apo2 ligand (TRAIL/Apo2L) in order to selectively induce apoptosis in tumor cells but not normal cells makes it an attractive target for development of new cancer therapy. Although TRAIL/Apo2L has been produced in several hosts, *E. coli* is one of the best expression systems among them due to its safety, simplicity, low cultivation cost, and known genetic properties. However, cytoplasmic expression of TRAIL/Apo2L in *E. coli* may be concomitant with some problems such as protease-induced degradation, protein misfolding, diminution in the biological activity and complexity of downstream processing. Therefore, the aim of this study was the development of an expression system to produce and secrete recombinant TRAIL/Apo2L into the *E. coli* periplasmic space. DNA encoding Alkaline Phosphatase (PhoA) signal peptide was added to the TRAIL/Apo2L cDNA using overlapping extension PCR procedure. PhoA-TRAIL construct was subsequently cloned in pET-22b expression plasmid and correct cloning was confirmed by PCR and sequencing. TRAIL/Apo2L expression was induced in *E. coli* BL21 (DE3) and then its periplasmic fraction was isolated through osmotic shock. SDS-PAGE analysis showed that recombinant TRAIL/Apo2L was successfully secreted into *E. coli* periplasm. The periplasmic TRAIL/Apo2L was identified by western blotting analysis. Finally, the biological activity of the purified periplasmic TRAIL/Apo2L was assessed by MTT assay to evaluate its growth inhibitory effect against the HeLa cell line. In conclusion, the results demonstrate that our TRAIL/Apo2L expression system could be an interesting alternative to reduce problems arose from the cytoplasmic production of TRAIL/Apo2L.

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Introduction

E. coli is the most commonly used host for the production of recombinant proteins [1]. As a gram negative bacterium, *E. coli* consists of two separate compartments: periplasmic and cytoplasmic space. While the majority of recombinant proteins have been produced in *E. coli* cytoplasmic space, it would seem that secretion of recombinant proteins into the periplasmic space has some considerable advantages over cytoplasmic production [2, 3]. Downstream processing steps of the periplasmic secreted proteins are much simpler because of these reasons: (i) contaminating proteins in the periplasm are lower than cytoplasm; (ii) formation of inclusion bodies (i.e. inactive protein aggregates) is minimal. The separation of recombinant protein from these inclusion bodies requires several refolding steps in order to obtain soluble and biologically active protein [4]; (iii) a simple method such as osmotic shock is only required for disruption of the outer membrane to release the target protein. In addition, the *E. coli* periplasmic space contains a number of chaperones and enzymes such as isomerases that promote the appropriate folding of recombinant proteins. Thus, periplasmic expressed proteins are mostly soluble and biologically active. Finally, secreted proteins into the periplasm are more stable as the protease activity of the periplasm is lower than the cytoplasm.

TNF related apoptosis inducing ligand (TRAIL/Apo2L) is a type II transmembrane protein which belongs to the TNF superfamily [5]. This ligand has some advantageous features over other TNF superfamily members such as TNF-α and Fas Ligand (FasL) [6]. Extracellular region of TRAIL/Apo2L has exhibited the strong apoptotic effect against several cancer cells but not normal cells [7]. Also, in contrast to other TNF superfamily members, TRAIL/Apo2L receptors are expressed in a wide range of tissues, suggesting that most tissues can be targets for TRAIL/Apo2L. For these reasons, TRAIL/Apo2L has become an attractive agent as anti-cancer therapy. Several studies have shown the anti-tumor activity as well as the safety of TRAIL/Apo2L pre-clinically [8, 9] and clinically [10, 11].

Although TRAIL/Apo2L has been produced in several hosts successfully [12-14], *E. coli* is the best expression system among them because of its safety, simplicity, low cultivation cost, and known genetic properties [1]. However, there are some problems concerning the cytoplasmic production of TRAIL/Apo2L in *E. coli* such as formation of inclusion bodies and downstream difficulties [15-17]. Therefore, considering the general advantages of protein secretion into the periplasmic space, the aim of this study was the development of an expression system in which a DNA encoding Alkaline Phosphatase (PhoA) signal peptide from *E. coli* was initially joined to TRAIL/Apo2L cDNA and then cloned in pET-22b plasmid in order to produce and promote the secretion of recombinant TRAIL/Apo2L into the periplasmic space of *E. coli*.

Materials and methods

Materials

Plasmids pET-22b and pTZ57R, and Ni-NTA resin were obtained from Novagen (U.S). Pfu DNA polymerase, restriction enzymes, DNA and protein ladder markers, and T4 DNA ligase were purchased from Fermentas (Lithuania). Primers were obtained from MWG (Germany). All antibodies were from Abcam (England) and nitrocellulose membrane was from Whatman (U.S). BCIP/NBT substrate system was from Sigma–Aldrich (Germany). HeLa cell line (ATCC Number: HTB-69) was purchased from Pasteur Institute (Iran). All other chemicals were obtained from Merck (Germany).

Vector construction

In order to insert N-terminal PhoA signal peptide and restriction sites into the TRAIL/Apo2L cDNA, Overlapping Extension Polymerase Chain Reaction (OE-PCR) method was performed using pfu DNA polymerase [18] (Figure 1). Three sequential PCR reactions were performed in a manner that the template used in each PCR reaction was the product of the previous PCR reaction. Also, almost 20 nucleotides of the 3′ region of each forward primer were similar to the 5′ region of the template in order to anneal with the template and then synthesizing the overhang sequence of forward primer (Table 1). Therefore, a small part of PhoA signal peptide was added to the 5′ region of the TRAIL/Apo2L cDNA in each PCR reaction runned as: pre-denaturation at 94°C for 2 min; first 5 cycles with denaturation at 94°C for 30 s, annealing at *65°C* for 30 s, and extension at 72°C for 2 min; followed by next 30

cycles of 94°C for 30 s, *69°C* for 30 s, and 72°C for 2 min; and post-extension by 72°C for 5 min.

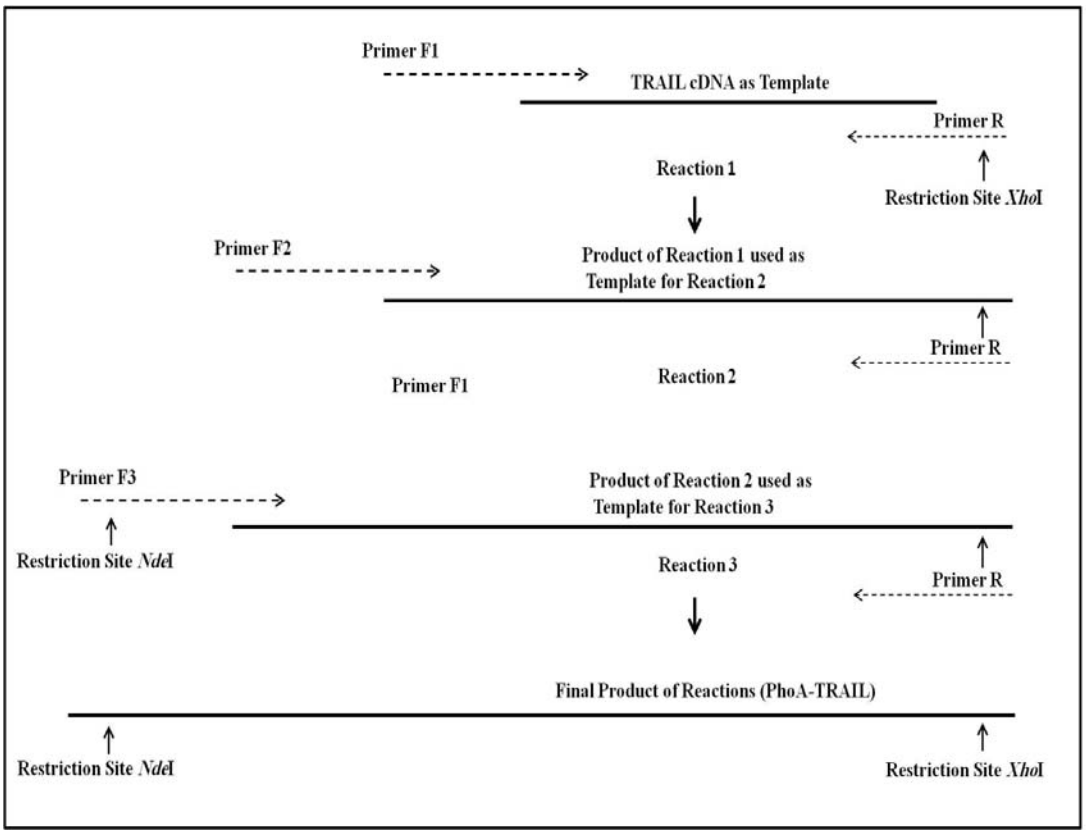

Fig. 1. OE-PCR procedure for the fusion of PhoA signal peptide to the TRAIL/Apo2L cDNA. Reaction 1: hybridization of primer F1 with terminal region of TRAIL/Apo2L cDNA and synthesis a part of PhoA signal peptide attached to TRAIL cDNA (F1-TRAIL: 546bp). Reaction 2: hybridization of primer F2 with F1-TRAIL fragment and synthesis an extended part of PhoA signal peptide attached to TRAIL cDNA (F2F1-TRAIL: 570bp). Reaction 3: hybridization of primer F3 with F2F1-TRAIL fragment and synthesis the whole PhoA signal peptide attached to TRAIL cDNA (PhoA-TRAIL: 594bp).

Table 1. Primers used for the synthesis of PhoA signal peptide at 5′ end of TRAIL/Apo2L cDNA.
a: F = Forward R = Reverse
b: Highlighted nucleotides of the F1, F2, and F3 primers are similar to the 5′ end region of the TRAIL cDNA, F1, and F2 primer, respectively. Highlighted nucleotides of R primer are similar to the 3′ end region of TRAIL cDNA. Restriction sites incorporated into primers are shown in italics.

[a]PrimerName	[b]Sequence	Restriction site
F1	5' CTGTTTACCCCGGTGACCAAAGCGGTGAGAGAAAGAGGTCCTCAG 3'	-
F2	5′ ATTGCCCTGGCCCTGTTACCTCTGCTGTTTACCCCGGTGACCAA 3'	-
F3	5′ AGGGGA*CATATG*AAACAAAGCACCATTGCCCTGGCCCTGTTACC 3'	*Nde*I
R	5′ AAAAAA*CTCGAG*TCCGCGTCCAACTAAAAAGGCCCCGAA 3′	*Xho*I

Subsequently, the PhoA-TRAIL/Apo2L fragment was at first purified and then cloned into the blunt-ended *Eco*RV restriction site of the pTZ57R cloning plasmid and then sub-cloned into the *Nde*I-*Xho*I restriction sites of the pET-22b expression plasmid under the control of T7 promoter. Also, cloning of TRAIL/Apo2L gene in this vector resulted in fusion of C-terminal consecutive 6 histidine residues (i.e. histag) which would facilitate the purification of soluble periplasmic TRAIL/Apo2L by Ni^{2+} affinity chromatography. Finally, correct cloning of the resulted construct, which was named PhoA-TRAIL/pET-22b, was confirmed by PCR and DNA sequencing.

Protein expression and cell fractionation

For transformation of recombinant plasmid PhoA-TRAIL/pET-22b into the *E. coli* BL21 (DE3) cells, $CaCl_2$ treatment followed by heating shock was performed [19] and the transformed cells were then cultivated in Terrific Broth (TB) medium (1.2% peptone, 2.4% yeast extract, 72 mM K_2HPO_4, 17 mM KH_2PO_4, and 0.4% glycerol) on a rotary shaker (200 rpm) at 37°C. After reaching the optical density of culture to 0.6, at 600 nm, isopropyl-β-D-thiogalacto-pyran-oside (IPTG) was added to a final concentration of 0.1 mM to induce the expression of the target protein. Cultivation was continued for further 24 h.

Thereafter, periplasmic and cytoplasmic fractions were separated using osmotic shock procedure according to the method of Khosla and Bailey [20]. In summary, cell pellet was obtained from 2 ml of culture (15000 g, 4ºC, 5 min) and resuspended in 2 ml of a hyper-osmotic solution (0.2M Tris; 200g/L sucrose; 0.1M EDTA; pH 8.0). After incubation on ice for 20 minutes, cells were harvested (15000 g, 4ºC, 15 min) and then resuspended in 2 ml of a hypo-osmotic solution (10mM Tris; 5mM $MgSO_4$; pH 8.0) followed by incubation on ice for 10 minutes. The resulting spheroplasts (i.e. cells without outer membrane) were pelleted (5000 g, 4ºC, 10 min) and the supernatant was kept as the periplasmic fraction. Finally, spheroplasts were resuspended in 2 ml of the hypo-osmotic solution and disrupted by ultrasonication in cycles of 20 s sonication and 20 s chilling on ice (dr.hielscher, Germany). The cycles were repeated until the solution was transparent. Cell debris was removed by centrifugation (15000 g, 10 min) and the supernatant was kept as the cytoplasmic fraction.

SDS-PAGE and Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Laemmli method [21]. In summary, protein samples were incubated with an equal volume of denaturing 2X sample buffer at 90°C for 5 minutes. Subsequently, proteins were separated under denaturing conditions using 5% stacking gel and 12% separating gel and then protein bands were Comassie-stained. Finally, bands intensity was measured by scanning the SDS-PAGE gel using Image J software (NIH).

For immunoblotting, the periplasmic proteins were separated by SDS-PAGE in 12% polyacrylamide gel and transferred to a nitrocellulose membrane using a semi-dry immunoblotting apparatus (Apelex, France). The membrane was blocked with 3% non-fat milk in TBS (10 mM Tris–HCl, 150 mM NaCl, pH 8.0) for 1 hour and subsequently washed three times for 5 minutes with TBST (TBS containing 0.05% Tween 20). The membrane was then incubated with 1:1000 anti-TRAIL/Apo2L polyclonal antibodies at room temperature for 2 hours. After washing three times for 10 minutes with TBST, the membrane was incubated with 1:1000 alkaline phosphatase conjugated secondary antibody at room temperature for 2 hours. Finally, the membrane was washed three times for 5 minutes with TBST and then western blotting was developed using the BCIP/NBT substrate system.

Purification of TRAIL/Apo2L

Ni^{2+} affinity chromatography was performed to purify periplasmic TRAIL/Apo2L [22]. In summary, according to the manufacturer's protocol, 2 ml of the 50% Ni-NTA His-bind resin slurry charged with Ni^{2+} was equilibrated with the binding buffer (50 mM NaH_2PO_4; 300 mM NaCl; 10 mM imidazole; pH 8). The periplasmic fraction solution was exchanged with binding buffer and loaded onto the column. Then, the column was washed twice with 4 ml of washing buffer (50 mM NaH_2PO_4; 300 mM NaCl; 20 mM imidazole; pH 8). Bound protein was subsequently eluted four times with 0.5 ml of eluting buffer (50 mM NaH_2PO_4; 300 mM NaCl; 500 mM

imidazole; pH 8). After elution, fractions were pooled, concentrated and buffer was exchanged against phosphate-buffered saline (PBS) and applied for further analysis. Protein sample concentrations were measured based on absorbance at 280 nm using a nanodrop spectrophotometer (Pharmacia biotech, Sweden).

Functional assay of TRAIL/Apo2L

Human cervical cancer HeLa cells were used to determine the effects of recombinant periplasmic TRAIL/Apo2L on cell proliferation. This cell line was prepared in Roswell Park Memorial Institute (RPMI) medium containing 10% Fetal Calf Serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin and then plated (2×10^3 cells/well) in 96-well plate with different concentrations of recombinant TRAIL/Apo2L ranged from 1 mg/L to 16 mg/L. PBS buffer and docetaxel (50 µM) was used as negative and positive control, respectively. The plate was incubated for 24 hours at 37°C in a humidified incubator with 5% CO2. After the incubation, cell growth inhibition was assessed by the MTT assay protocol as previously described [23].

Results and Discussion

Construction of PhoA-TRAIL/pET-22b expression vector

The fusion of a proper N-terminal signal peptide to a recombinant protein could conduct it into the *E. coli* periplasmic space [24, 25]. Considering the high ability of PhoA signal peptide for transporting a large amount of Alkaline Phosphatase into the *E. coli* periplasmic space, this signal peptide has been frequently used in several studies for manipulation of the secretion pattern of various recombinant proteins expressed by *E. coli* [26, 27].

DNA encoding the PhoA signal peptide was added to 5' end of the TRAIL/Apo2L cDNA using three sequential OE-PCR reactions (Figure 1). As anticipated, gradual increase in the size of the PCR products to a final 594 bp fragment indicated the insertion of DNA sequence encoding PhoA signal peptide to TRAIL/Apo2L cDNA (Figure 2). During each PCR reaction, the 5' overhang region of the forward primer was added to the DNA template after annealing its 3' region with 5' end of the DNA template. For this reason, the size of each PCR product was increased proportional to the length of the primer overhang sequence.

As seen in the method section, annealing temperature of the first five cycles of each PCR was adjusted lower than next thirty cycles. This is because while, in the first cycles of the PCR reaction, the forward primer was hybridized to the template with only its 3' overlapping end region, in the later cycles, hybridization occurred with whole primer length.

It is noteworthy that since the template of each PCR reaction was the product of the previous PCR reaction, it is likely to insert incorrect nucleotides into the final PCR product. Therefore, pfu DNA polymerase with proofreading ability was used to reduce the probable insertion of such a wrong nucleotide into the final PCR product.

Subsequently, the final PCR product was firstly cloned into blunt-ended *EcoR*V restriction site of pTZ-57R cloning vector and then sub-cloned into *Nde*I and *Xho*I restriction sites of the pET-22b expression vector. Finally, the correct insertion of PhoA-TRAIL/Apo2L fragment into the plasmid was confirmed by PCR analysis and DNA sequencing.

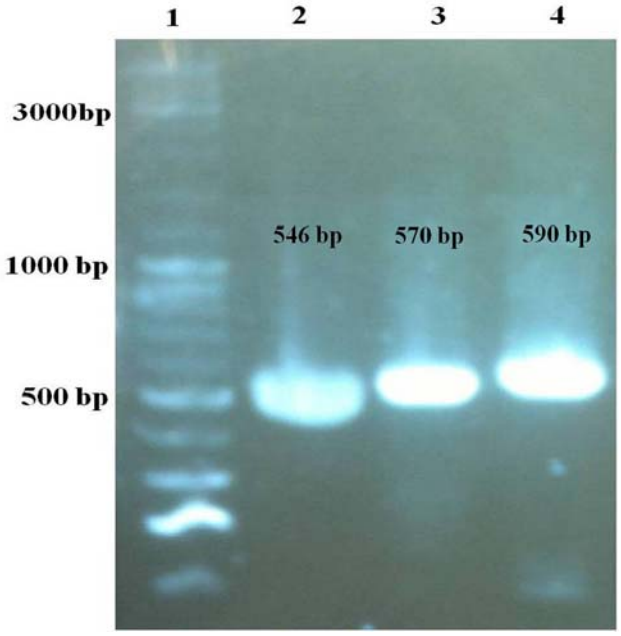

Fig. 2. Gel electrophoresis analysis of OE-PCR products. Lane 1: DNA Ladder marker. Lane 2: 546 bp product amplified with F1R primer resulting in F1-TRAIL fragment. Lane 3: 570 bp product amplified with F2R primer resulting in F2F1-TRAIL fragment. Lane 4: 590 bp product amplified with F3R primer resulting in PhoA-TRAIL whole sequence.

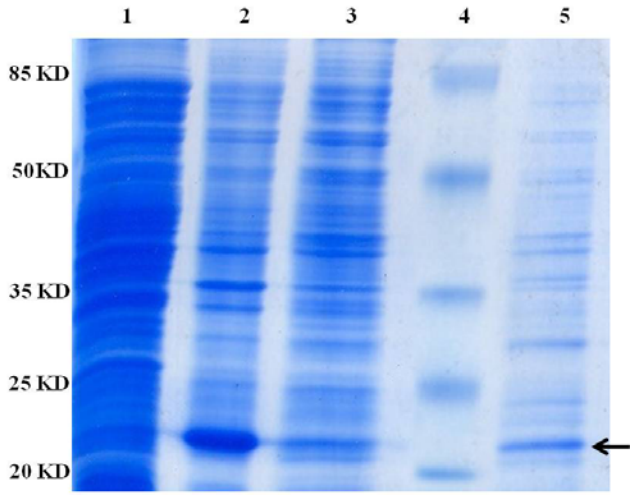

Fig. 3. SDS-PAGE analysis of sub-cellular fractions of *E. coli* BL21 (DE3) expressing PhoA-TRAIL. Lane 1: whole cell before induction. Lane 2: whole cell after induction with a 21 KDa band indicating recombinant TRAIL. Lane 3: cytoplasmic fraction after induction. Lane 4: protein marker. Lane 5: periplasmic fraction after induction. The arrow indicates periplasmic secreted TRAIL.

Expression of periplasmic TRAIL/Apo2L

In order to identify whether recombinant plasmid PhoA-TRAIL/pET-22b was able to express and secrete recombinant TRAIL/Apo2L into periplasmic space, protein expression was firstly induced in the *E. coli* BL21 (DE3) and then different fractions was separated based on osmotic shock procedure.

SDS-PAGE analysis of periplasmic and cytoplasmic fractions revealed the high level expression of TRAIL/Apo2L. Considering the bands intensities, it could be concluded that almost 36% of total expressed TRAIL/Apo2L was secreted into the periplasmic space (Figure 3). Several mechanisms have been proposed for protein secretion into the periplasmic space [28]. The N-terminal PhoA signal peptide interacts with Sec-dependent secretion pathway agents which is the most efficient secretion pathway [29]. New synthesized polypeptide is recognized by SecB chaperone and subsequently directed to the membrane bound secretion machinery where the protein is translocated into the periplasmic space in its unfolded state. During this process, the signal peptide is cleaved and the mature protein is folded by periplasmic chaperones. However, due to the limited capacity of the secretory pathway, high rate of TRAIL/Apo2L expression may lead to accumulation inside the cell as an inclusion body. Therefore, a low concentration of IPTG (0.1 mM) was added to culture medium in order to reduce the expression rate.

Immunological identification and purification of periplasmic TRAIL/Apo2L

The identity of periplasmic TRAIL/Apo2L was confirmed by western blotting analysis using anti-human TRAIL/Apo2L antibody. The size of the protein band on the nitrocellulose membrane was estimated 21 KDa and could be attributed to the recombinant TRAIL/Apo2L (Figure 4).

The major advantage of periplasmic secretion is lower amounts of contaminating proteins than cytoplasm but periplasmic TRAIL/Apo2L should be purified in order to analyze its biological function. As shown in Figure 5 recombinant TRAIL/Apo2L was purified effectively in which other contaminating proteins were successfully removed. Expression vector pET-22b possesses a DNA sequence located in downstream region of the multiple cloning site (MCS) which results in fusion of a histag to the C-terminal region of recombinant protein after expression. Using this histag, recombinant TRAIL/Apo2L was purified with Ni-NTA affinity chromatography method. In this procedure, the interaction between imidazole side chains of the recombinant TRAIL/Apo2L histag and Ni^{2+} ion attached to column results in retention of recombinant TRAIL/Apo2L while unbounded contaminating proteins are washed away. In elution step, recombinant TRAIL/Apo2L will dissociate from the Ni-NTA column by increasing the imidazole concentration which is the mildest elution method for purifying native proteins.

Since high concentration of imidazole may interfere with the assessment of TRAIL/Apo2L biological function, any interfering components such as imidazole were removed by dialysis against PBS buffer over night.

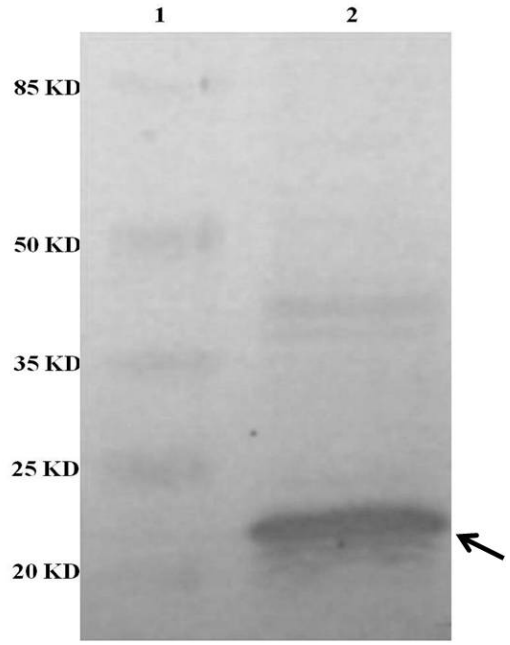

Fig. 4. Western Blot analysis for identification of periplasmic secreted TRAIL. Lane 1: protein marker. Lane 2: periplasmic fraction representing a 21 KDa band shown by an arrow as expected for TRAIL/Apo2L.

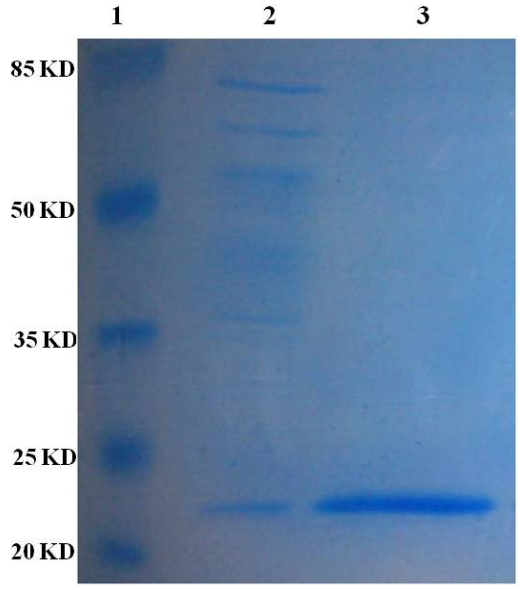

Fig. 5. SDS-PAGE analysis of purification of periplasmic secreted TRAIL by Ni-NTA affinity chromatography. Lane 1: protein marker. Lane 2: periplasmic fraction of *E. coli* BL21 (DE3) after induction. Lane 3: purified periplasmic TRAIL after elution by imidazole indicating approximately 100% purity using ImageJ software (NIH).

Functional assay of periplasmic TRAIL/Apo2L

MTT assay was performed in order to analyze the growth inhibitory effect of periplasmic expressed TRAIL/Apo2L on cancerous HeLa cell line which is a common cell line in pharmaceutical research studies for analyzing the anti-tumor activity [30]. Periplasmic expressed TRAIL/Apo2L reduced the growth of HeLa cells in a dose-dependent manner (Figure 6). Growth inhibitory effect of periplasmic TRAIL/Apo2L in this study is consistent with previous results [14, 31]. However, in those studies, recombinant TRAIL was produced in *E. coli* cytoplasm as inclusion body and needed to be refolded to obtain biological effect, while, in the present study, periplasmic TRAIL was soluble and biologically active.

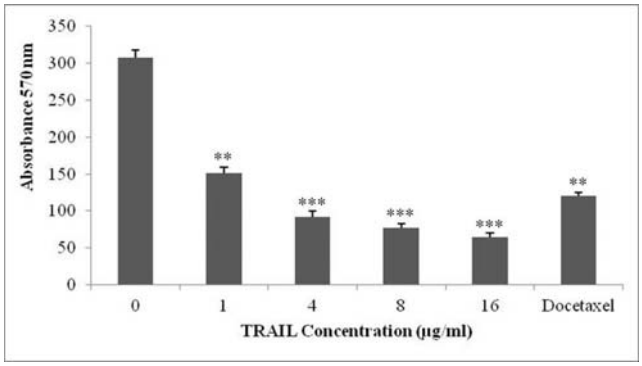

Fig. 6. Growth inhibitory effect of periplasmic TRAIL/Apo2L on human cervical cancer HeLa cells. HeLa cells were transferred into 96-well plate and treated with different concentrations of TRAIL/Apo2L for 24 h. PBS buffer and docetaxel (50 µM) was used as negative and positive control, respectively. The inhibitory effect on cell growth was then assessed using MTT assay procedure. Each bar indicates the mean ± SD of three independent experiments and analyzed for statistical significance using ANOVA followed by Scheffe's test for multiple comparison. **p < 0.01 and ***p < 0.001 significantly different from negative control.

Conclusion

The present study has introduced an expression system based on PhoA signal peptide and pET-22b plasmid which could effectively produce and secrete recombinant TRAIL/Apo2L into *E. coli* periplasm. Considering the advantages of secreting recombinant TRAIL/Apo2L into the *E. coli* periplasmic space, this study may be useful in presenting a new approach for

production of this potential anticancer therapeutic agent.

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Conflict of Interest

Authors certify that no actual or potential conflict of interest in relation to this article exists.

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