ISSN (Online): 2091-2609
DOI Prefix: 10.3126/ijasbt
International Journal of Applied Sciences and Biotechnology
(IJASBT)

Research Article

Cloning and Expression of Alpha Hemolysin Toxin Gene of *Staphylococcus aureus* Against Human Cancer Tissue

Gamal M. Abdel-Fattah[1], Elsayed E. Hafez[2], Mysaa E. Zaki[3] and Nada M. Darwesh[2*]

[1]Botany Department, Faculty of Science, Mansoura University, Egypt
[2]City of Scientific Research and Technological Applications, Borg El Arab, Egypt
[3]Clinical pathology, Faculty of Medicine, Mansoura University, Egypt

*Corresponding author's email: nadadarweshmoh@gmail.com

Abstract

Recombinant technology has crucial impact in therapy development. In microbial environment, Pathogenic bacteria such as *Staphylococcus aureus* produces alpha hemolysin protein. This protein is used as anticancer protein. In our study, alpha hemolysin toxin (*hia*) gene was isolated from *S. aureus* isolate. The isolate of *S. aureus* was isolated from blood samples of patients at Microbiology laboratory of Chlidren Mansoura Unversity Hospital. The purified PCR of *hia* gene of *S. aureus* strain was subjected to sequencing and cloning. Recombinant alpha hemolysin toxin cloning was detected by PCR, and α- hemolysin protein was purified by Sphadex. The purified α-protein was used as anticancer against HepG-2 cells (human Hepatocellular carcinoma), HCT-116 cells (human colon carcinoma), MCF-7 cells (human breast cancer cell line) and A-549 cells (human Lung cancer cell line).

Keywords: *Staphylococcus aureus;* Alpha hemolysin toxin; Sequencing; Cloning; Anticancer tissue.

Abbreviations: *hia*: Alpha hemolysin toxin; PCR: Polymerase Chain Reaction; HepG-2 cells: human Hepatocellular carcinoma; HCT-116 cells: human colon carcinoma; MCF-7 cells: human breast cancer cell-line; A-549 cells: human Lung cancer cell line.

Introduction

In therapeutic system, *Staphylococcus aureus* play crucial role in pathogenic world (Reddy *et al.*, 2014). *S. aureus* is faculititive human pathogenic that causes a wide range of infections from skin and soft tissue to invasive such as endocarditis, osteomyelitis, and pneumonia (Bartlett and Hulten, 2010; Aman and Adhikari, 2014; Borrello *et al.*, 2007).

Pathogenic *S. aureus* have a wide range of virulence factors as superantigens (enterotoxins, toxic shock syndrome toxin and exfoliative toxins), cytotoxins (alpha-hemolysin, beta-hemolysin, gamma-hemolysin, delta-hemolysin, Panton-Valentine leukocidin), phagocytosis inhibitors (polysaccharide capsule, protein A), and immune evasion molecules (chemotaxis inhibitory protein, staphylokinase, aureolysin) (Bartlett and Hulten, 2010; Aman and Adhikari, 2014; Borrello *et al.*, 2007).

Alpha hemolysin toxin of *S. aureus* (*hia*) is monomer water-soluble 34 KDa exotoxin protein of 293 amino acids and chromosomally gene. Alpha-toxin is a pore-forming hemolytic toxin that brings about membrane damage to many types of mammalian cells. Alpha hemolysin toxin can be increased the productivity by cloning (Leng *et al.*, 2011; Tavares *et al*., 2014; Gurnev and Netorovich, 2014; Kong *et al.*, 2016). Alpha hemolysin toxin plays a role in inducing apoptosis in tumor cells, so it can be used as anticancer (Wang *et al.*, 2011).

In this connection, the main objective of this paper is to isolate and identify the most effective isolate of *S. aureus* for producing α- hemolysin toxins and more deeply to evaluate the potential efficacy of this toxins against cancer of human cells tissues.

Materials and Methods

Collection and isolation of S. aureus isolate

S. aureus isolate used in this study was isolated from blood samples of patients at Chlidren Mansoura University Hospital (CMUH). *S. aureus* isolate was identified according to morphological and biochemical identification according to methods of Sogaard *et al*. (2007) and Rubin *et al*. (2010).

Isolation of Alpha Hemolysin Toxin of S. aureus

Primer

hia primer sequences was selected as Tavares (2014): RT_Forward: 5'-TAATGAATCCTGTCGCTAATGCC-3', RT_reverse: 5'-CACCTGTTTTTACTGTAGTATTGCTTCC-3'.

Bacterial Suspension Preparation

S. aureus isolate used in this study was grown in 5 ml of trypton soya bean (TSB) broth at 37 ºC for 24 hours then the pellets were collected by centrifugation at 12000 rpm for 5 minutes according the method of Liang *et al.* (2011). The cell pellets were stocked at 4ºC until used.

RNA Extraction by Kit Method

RNA isolation from bacterial cells was carried out using the protocol of RNAasy kit (Intronbio- technology, Korea).

Preparation of Complementary Deoxyribonucleic acid (cDNA)

The cDNA reaction was performed using reverse transcriptase enzyme. The reaction was carried out in a 20 µl reaction mixture containing: 3 µl of RNA extraction, 2.5 µl of buffer, 2.5 µl of dNTPs (dATP, dCTP, dGTP and dTTP), 5 µl of the primer (reverse primer of specific *hia* gene), 0.2 µl of reverse transcriptase enzyme, 6.8 µl of PCR water (nuclease – free). The tubes containing reaction mixture were mixed, and transferred to the PCR apparatus and the program was entered as the following: The reaction was initiated by first step at 42ºC for 1 hour, followed by second step at 72º for 10 mintes. cDNA products were stored at -20ºC until used.

25 µl of reaction mixture PCR reaction was made with 2.5 µl of stored cDNA, 5 µl of buffer, 4 µl of M $MgCl_2$, 3 µl of dNTPs (2.5 mM of each), 3 µl of the primer (1.5 µl of forward + 1.5µl of reverse), 0.4 µl of Taq DNA polymerase (Promega, U.S.A.), 7.1 µl of PCR water (nuclease–free). The tubes containing reaction mixture were vortexed, and transferred to the PCR-apparatus. The thermal program was run as follows: The initial denaturing step at 95ºC for 2 minutes, followed by 30 cycles of denaturation at 95ºC for 1 minute, annealing at 50ºC for 1 minute and extension at 72ºC for 1 minutes and finally the reaction was terminated with an extension step at 72ºC for 5 minutes. At the final, the program was stopped. Amplified products were electrophoresed in 2% agarose with ethidium bromide, photographed and analyzed (Tavares, 2014; Ouyang *et al.*, 2016).

PCR product Purification

PCR product of α-toxin gene was purified by using DNA gel extraction kit (Biobasic Inc, Canada). Purified DNA was stored at -20°C until used in gene sequencing and gene cloning expression.

Sequence Analysis of Alpha Hemolysin Toxin of S. aureus

The alpha hemolysin toxin specific PCR products of the strain *S. aureus* was sequenced using automated DNA sequence by Macrogene (Macrogene com, Korea). The obtained DNA analysis using comparative tool such as Blast DNA (www.ncbi.nlm.nih.gov) (Rajendhran and Gunasekaran, 2010).

Cloning of S. aureus Alpha Hemolysin Toxin

The purified PCR product of *S. aureus* and expression vector (pH6HTN His6HaloTagT7) (Promega, U.S.A.) were treated with the same suitable restriction enzyme (*EcoRI*) (Promega, U.S.A.). Restriction enzyme was chosen by using the sequence of purified PCR of *S. aureus* in online site (www.restrictionmapping.com). The restricted purified PCR product was ligated with the restricted vector by using T4 DNA ligase enzyme (Promega, U.S.A.). Cloned vector was transmitted into competent *E. coli* DH5α cells. Transformed cells were identified by the colour of colonies using antibiotic (ampicillin) containing medium with presence of X-gal and IPTG (isopopropyl B-D-1-thiogalactopyranoside). The above methods have been described by Inoue *et al.* (1990), Reddy *et al.* (2014) and Pulicherla *et al.* (2013).

Detection of Gene Cloning by using PCR

Plasmid DNA Isolation

White colony was inoculated in 5 ml of Luria Bertani (LB) medium supplemented with Ampicillin (50mg/ ml) and incubated at 37 ºC for 24 hours in shacking condition (Pulicherla *et al.,* 2013). The bacterial cells were harvested by using centrifuge at 12000 rpm for 10 minutes. Plasmid was extracted by using extraction plasmid kit (Biobasic Inc, made in Canada). The plasmid DNA was eluted by adding 30 µl of elution buffer and the plasmid stored at -20°C until used.

PCR for Isolated Plasmid

PCR reaction was same as described above for PCR of *S. aureus*.

Recombinant Alpha Hemolysin Toxin Extraction and Purification

The sample preparation and the protein purification were done by the methods of Reddy *et al.* (2014). Expression of *hia* gene and separation of *hia* protein were carried out by using SDS-PAGE. White colonies were cultured in 200 ml of LB broth medium with inducer IPTG and ampicillin for 24 hours. The culture was centrifuged at 10000 rpm for 30 minutes in cooling centrifuge. The harvested pelletes were suspened with 25 ml of gel permission buffer and sonicated on ice for 5 minutes, and then centrifuged at 10000 rpm for 20 minutes. The supernatants were collected in a new sterilized fluken tube and transferred to sphadex column for protein purification (El-Gayar, 2015). The protein fractionated was taken 1 ml per 1 minute. The obtained α-toxin protein were determined by using method of Bradford (1976). The purified protein SDS-PAGE is shown in (Fig. 4C) which was performed the methods of Harlow and lane (1988). The purified α- protein was dried by lyphiliziation.

Application of Recombinant A- Hemolysin Protein In Different Human Cancer Tissues

Mammalian cell lines

HepG-2 cells (human Hepatocellular carcinoma), HCT-116 (colon carcinoma), MCF-7 cells (human breast cancer cell line) and A-549 cells (human Lung cancer cell line) were obtained from VACSERA Tissue Culture Unit.

Cell lines Propagation

The cell lines (HepG-2, HCT-116, MCF-7, A-549) propagation were described by *Mosmann* (1983).

Cytotoxicity of Alpha-Hemolysin Protein Using Viability Assay

For cytotoxicity assay, the cells were seeded in 96-well plate at a cell concentration of 1×10^4 cells per well in 100µl of growth medium. Fresh medium containing different concentrations of recombinant alpha hemoysin protein was added after 24 h of seeding. Serial two-fold dilutions of the tested α- protein were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiter plates (Falcon, NJ, USA). The microtiter plates were incubated at 37ºC in a humidified incubator with 5% CO_2 for a period of 48 h. Three wells were used for each concentration of α-protein. Control cells were incubated without test sample and with or without DMSO. After incubation of the cells for at 37°C, various concentrations of α- hemolysin protein were added, and the incubation was continued for 24 h and viable cells yield was determined by a colorimetric method which the cells were stained by the crystal violet stain (Mosmann, 1983; Gomha *et al.*, 2015).

The relation between surviving cells and α-toxin concentration is plotted to get the survival curve of each tumor cell line after treatment with α-toxin. The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose response curve for each conc. using Graphpad Prism software (San Diego, CA. USA) (Mosmann, 1983; Gomha *et al.*, 2015).

Results and Discussion

Alpha hemolysin toxin of *S. aureus* is one of the most important virulence factors. *hia* gene of *S. aureus* used in this study was detected and isolated by using PCR. Isolated α- toxin gene was subjected to sequencing and cloning. Alpha hemolysin protein was generated from recombinant *hia* gene in competent *E. coli* cells. The purified protein was treated human cancer cell lines.

Isolation and Identification of S. aureus Isolate

The isolate produced yellow colonies when grown on blood agar medium as shown in Fig. (1-A). This isolate was Gram-positive cocci shaped, coagulase positive. According, this strain was prelimerary identified to be *S. aureus*. Alpha hemolysin expression was detected on blood agar medium, which the color turned to brownish color as showed in Fig. (1B). These results showed agreement with those published by Reddy *et al.* (2014); Stulik *et al.* (2014).

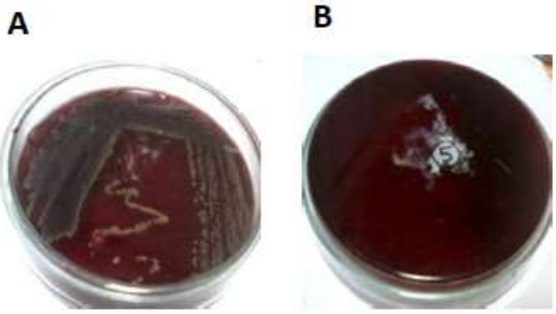

Fig. 1: A) Yellow colonies of *S. aureus* on blood agar. B) *S. aureus* was produced alpha hemolysin toxin on blood agar medium which turned to brownish color.

Analysis of Sequence of Alpha Hemolysin Toxin of S. aureus

α-toxin gene (*hia*) and purified *hia gene* were exactly 680 bp as showed in Fig.2. Purified *hia* gene was sequenced as Fig. 3. The Purified PCR product was sequenced by Microgene (in Korea) then the sequences were submitted to NCBI, and Blast sequence analysis showed that α-toxin has been 99% according with sequences publishted in Gene Bank. This result is agreement with this published by Fei *et al.* (2011).

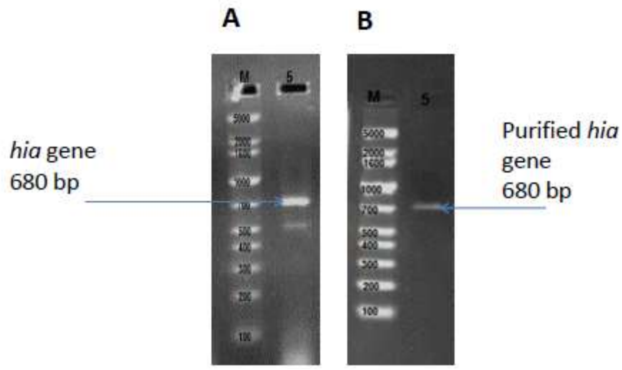

Fig. 2: A) Amplified PCR of *hia* gene of *S. aureus* from blood, with the amplicon size of exactly 680 bp. B) Purified α-toxin of *S. aureus* strain. Amplified PCR of *hia* gene was ran in 2% of agarose gel and then bands were eluted by the gel elution kit. M: 1000 bp DNA marker (100, 200, 300, 400, 500, 700, 1000, 1600, 2000, 5000).

CCTGATGCGAGAGTGCTACAAAGTGGTTTAGCCTGGCCTTCAGCCTTTAAGGTACAGTT
GCAACTACCTGATAATGAAGTAGCTCAAATATCTGATTACTATCCAAGAAATTCGATTGAT
ACAAAAGAGTATATGAGTACTTTAACTTATGGATTCAACGGTAATGTTACTGGTGATGAT
ACAGGAAAAATTGGCGGCCTTATTGGTGCAAATGTTTCGATTGGTCATACACTGAAATAT
GTTCAACCTGATTTCAAAACAATTTTAGAGAGCCCAACTGATAAAAAAGTAGGCTGGAA
AGTGATATTTAACAATATGGTGAATCAAAATTGGGGACCATATGATAGAGATTCTTGGAA
CCCGGTATATGGCAATCAACTTTTCATGAAAACTAGAAATGGTTCTATGAAAGCAGCAGA
TAACTTCCTTGATCCTAACAAAGCAAGTTCTCTATTATCTTCAGGGTTTTCACCAGACTT
CGCTACAGTTATTACTATGGATAGAAAAGCATCCAAACAACAAACAAATATAGATGTAAT
ATACGAACGAGTTCGTGATGATTACCAATTGCATTGGACTTCAACAAATTGGAAAGGTA
CCAATACTAAAGATAAATGGACAGATCGTTCTTCAGAAAGATATAAAATTCGATTGGGA

Fig. 3: Sequence of alpha hemolysin toxin of *S. aureus* form PCR product. Amplified gene was isolated from agarose gel and sequenced to confirm the present of *hia* gene of *S. aureus*.

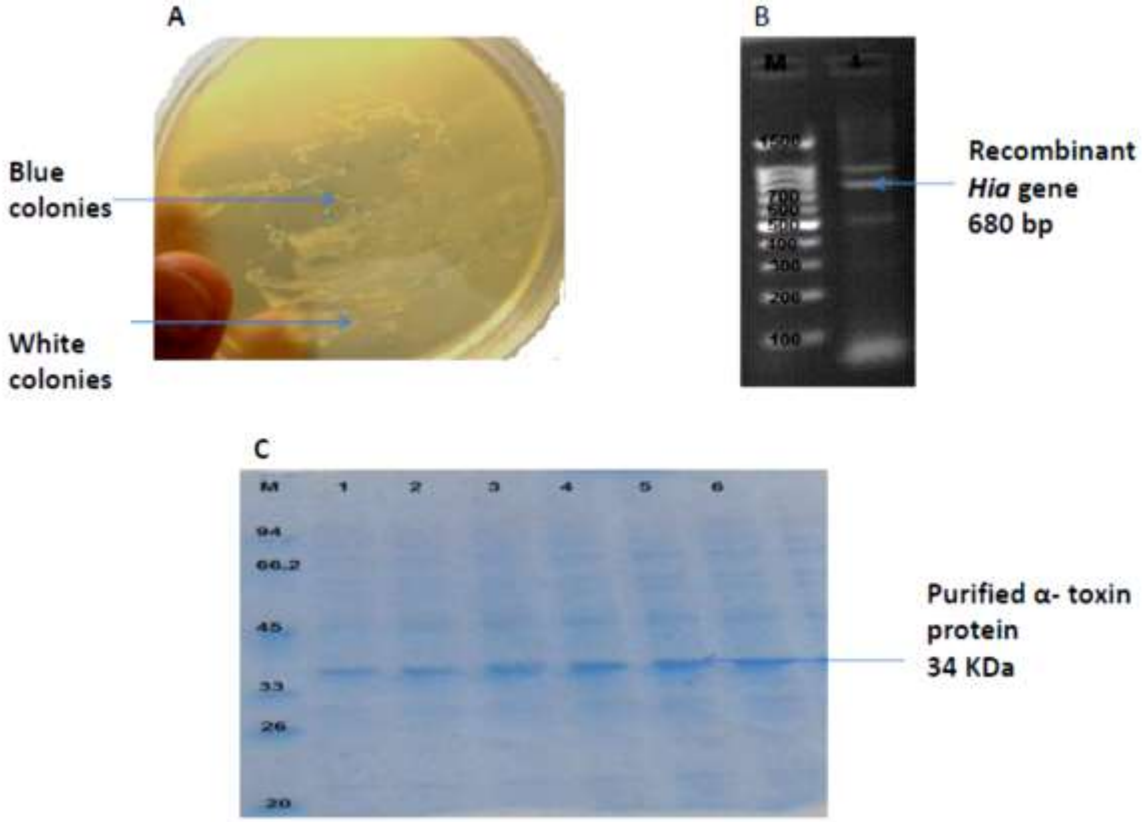

Fig. 4: Expression of recombinant *hia* protein from recombinant *E. coli*. A) The recombinant *E.coli* colonies on L. B. medium. The white colonies were detected to the gene insertion while the blue colonies were not inserted the gene. The wihte colonies were selected. B): PCR-detection of alpha hemolysin toxin after cloning. PCR of purified plasmid after cloning. M: M: 1000 bp DNA marker. C): Purified alpha hemolysin toxin (1- 6 purified fractional protein) after cloning in *E. coli*. M: Marker protein (94, 66.2, 45, 33, 26, 20).

Cloning of Alpha Hemolysin Toxin Gene Into Ph6htnhishalotagt7 Expression Vector

The PCR product of α- toxin of *S. aureus* was separated on 2 % agarose gel and purified PCR using gel extraction kit (thermo). The purified PCR and the expression vector were cut with the same restriction enzyme (*Eco*R1) then ligated with ligase enzyme (promega). The recombinant vector was transformed into *E.coli* DH5α and white colonies were selected (Fig. 4-A). Plasmid DNA min prep form the white colonies were performed using plasmid isolation kit. The purified plasmid was used in PCR test for detection of α-toxin gene (Fig.4-B). The recombinant bacteria were induced in L. B. broth medium using IPTG and Ampecillin and the production of alpha hemolysin toxin was evaluated. The recombinant alpha hemolysin toxin was purified by using sphadex column and the obtained α-toxin were determined by using Bradford method. The activity of all obtain fraction were screened and separated by using SDS-PAGE as showed in the Fig. (4-C). α-toxin protein was expressed at 34 KDa and this result is agreement with those published by Bantel *et al.* (2001) and Swofford *et al.* (2014).

Application of Recombinant A- Hemolysin Protein in Different Human Cancer Tissues

Purified recombinant alpha hemolysin obtained during this study was dried by lyphiliziation. Cytoxicity of recombinant alpha hemolysin protein was tested against four cell lines such as HepG-2, HCT-116, MCF-7, A-549.

In this study showed that different concentrations of alpha hemolysin toxin was effected on the cell viability of four cell lines (HepG-2, HCT-116, MCF-7, A-549) and determined IC50 under these experimental conditions. From Fig. 5 and Fig. 7A, inhibitory activity of α-toxin against Hepatocellular carcinoma cells was detected with IC50 = 210 µg/ml. From Fig. 6 and Fig. 7B, inhibitory activity of α-toxin against colon carcinoma cells was detected with IC50 = 244 µg/ml. From Fig. 7C and Fig. 7D, weak inhibitory activity of alpha toxin against Breast carcinoma cells and Lung carcinoma cells were detected with IC50 = >500 µg/ml. Our results confirmed that alpha hemolysin toxin is used as anticancer. These results are agreement with those published by Bantel *et al.* (2001); Swofford *et al.* (2014). In this connection, Vandenesch *et al.* (2012) has reported that α-hemolysin is the most characterized virulence factor of *S. aureus*. Upon binding to the cell surface, α-hemolysin monomers assemble into a homo heptamer, forming a prepore. The prepore subsequently transitions to a mature β-barrel transmembrane pore. This pore allows the transport of molecules smaller than 2kD such as K+ and Ca2+ ions, leading to necrotic death of the target cell

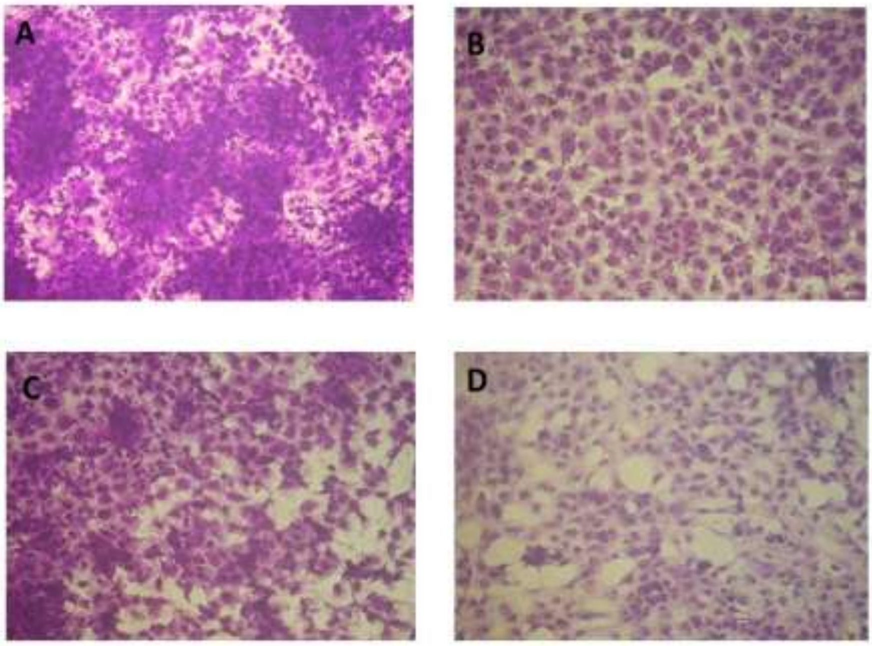

Fig. 5: Microscopic examination for the effect of α-hemolysin on hepatocellular cancer cells (HepG-2). The cells were incubated with (A) 0, (B) 62.5, (C) 250 or (D) 500 µg/ml of α-hemolysin at 37°C for 48 hours. After that, the cell viability was determined by using 1% crystal violet stain.

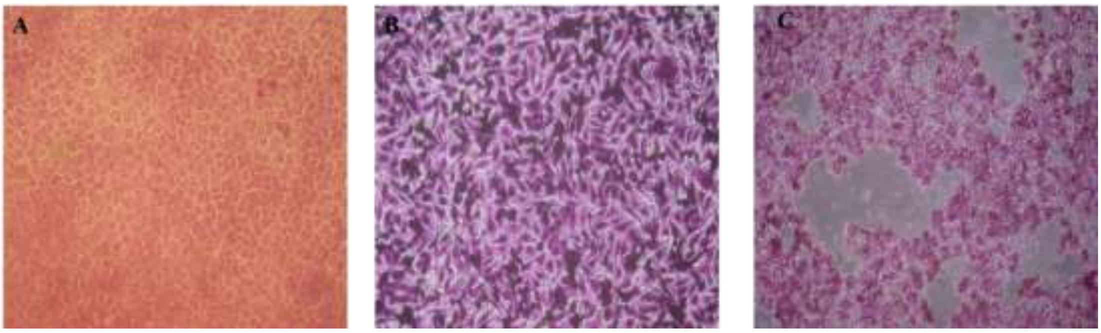

Fig. 6: Microscopic examination for the effect of α-hemolysin on colon cancer cells (HCT-116). The cells were incubated with (A) 0, (B) 62.5 or (C) 500 µg/ml of α-hemolysin at 37°C for 48 hours. After that, the cell viability was determined by using 1% crystal violet stain.

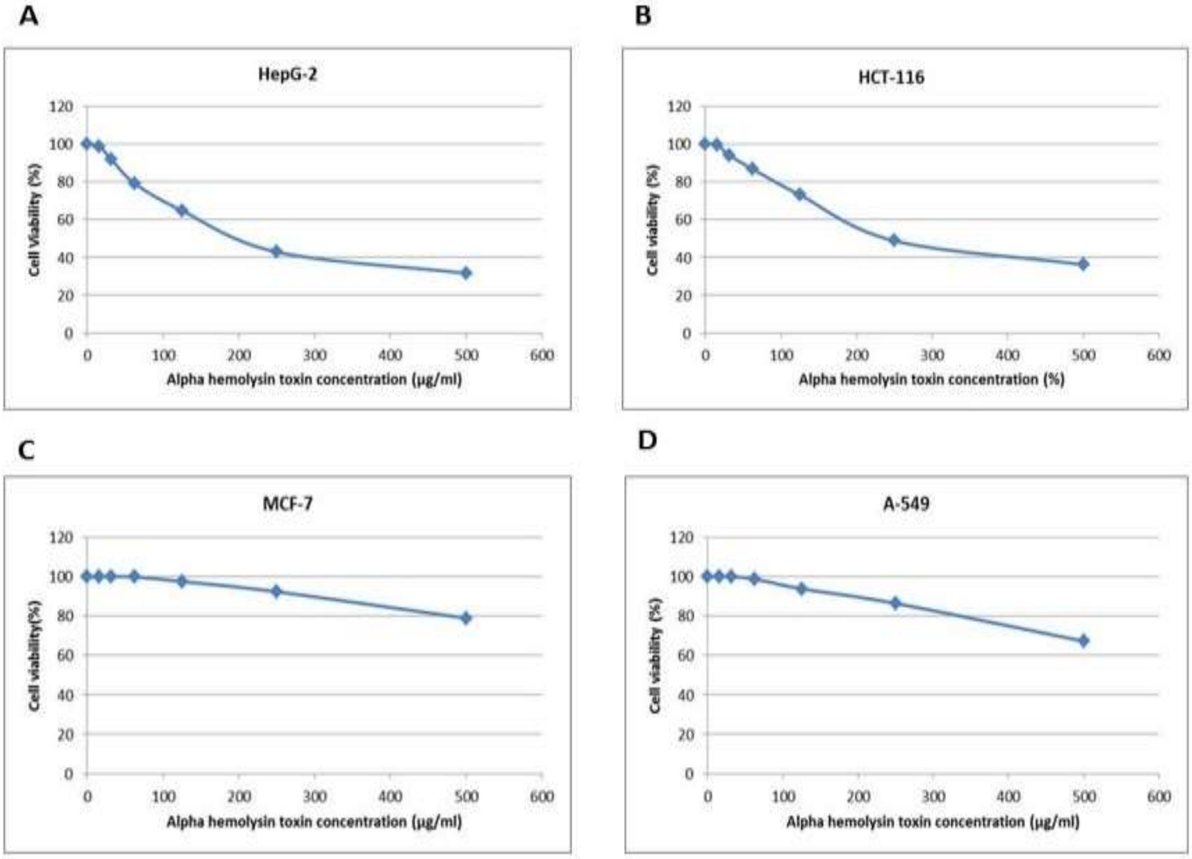

Fig. 7: cytotoxicity of α-hemolysin against hepatocellular cancer, colon cancer, Breast cancer and Lung cancer cell lines. (A) hepatocellular cancer cells [HepG2], (B) colon cancer cells [HCT-116],(C) Breast cancer cells [MCF-7] and (D) Lung cancer cells [A-549] were treated with serial dilutions of α-hemolysin for 48 hours then the percentage of viable cells after treatment was determined by using crystal violet.

Table 1: Crystal violet staining percentage of viability of human cancer cells.

Cell line	**Cell viability (%)**
Human hepatocellular cancer cell line (HepG-2)	31.69
Human colon cancer cell line (HCT-116)	36.27
Human breast cancer cell line (MCF-7)	78.95
Human lung cancer cell line (A-549)	67.28

In this study, that high concentration of α-toxin (500 µl/ ml) was effected on the cell viability of four cells line (HepG-2, HCT-116, MCF-7, A-549) as showed in Table (1). The cell viability of cell lines were represented 31.69% in HepG-2, 36.27% in HCT-116, 78.95% in MCF-7, 67.28% in A-549. From our results, Alpha hemolysin protein had high inhibitory activity against HepG-2 and HCT-116, and had weak inhibitory activity against MCF-7 and A-549. In this connection, Haslinger *et al.* (2003); Essmann *et al.* (2003) had reported that *S. aureus* α- toxin is a pore-forming toxin that can caused apoptosis of tumor cells at higher concentrations. In this study, it was found that the cell viability of MCF-7 was represented 78.95% per 48 hours. Dissimilar results were reported by Swofford *et al.* (2014) where the cell viability represented 85% in less 1 h of in human breast cancer (MCF-7). In this study, the cell viability of A-549 was represented 67.28%. This result of lung cancer cell line is agreement with Johansson *et al.* (2008).

Conclusions

Alpha hemolysin protein was isolated from pathogenic *S. aureus* that was isolated from blood samples of patients (chrildren). This recombinant *hia* gene was produced alpha hemolysin protein by using competent *E. coli* as a host. The recombinant α- toxin protein was used as anticancer agent. The production of recombinant α- toxin was very simple process, low cost, high efficiency, non-toxic, high yield and no side effect.

References

Aman MJ and Adhikari RP (2014) Staphylococcal biocomponent pore-forming toxins: targets for prophylaxis and immuno-therapy. *Toxins* **6**(3): 950-972. DOI: 10.3390/toxins6030950

Bantel H, Sinha B, Domschke W, Peters G, Schulze-Osthoff K and Janicke RU (2001) α-toxin is mediator of *Staphylococcus* *aureus*-induced cell death and activates caspases via the intrinsic death pathway independently of death receptor signalling. *The journal of cell biology* **155**(4): 637-648. DOI: 10.1083/jcb.200105081

Bartlett AH and Hulten KG (2010) *Staphylococcus aureus* pathogenesis. *The pediatric infectious Disease journal* **29**: 860-861. DOI: 10.1097/INF.0b013e3181ef2477

Bordford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Annu Rev Biochem* **72**: 248-254. DOI: 101016/0003-2697(76) 90527-3

Borrello SP, Murray PR, Funke G (2007) Chapter 14: Staphlycocci Toplelyand wilson's microbiologyand microbial infections, Bacteriology (3th eds) 2-367 Saunders, an imprint of Elsevier Inc

El-Gayar KE (2015) Principles of recombinant protein production, extraction and purification from bacterial strains. *Inter J Microbiology and Allied sciences* **2**: 18

Essmann F, Bantel H, Totzke G, Engels IH, Sinha B, Schulze-Osthoff K and Janicke RU (2003) *Staphylococcus aureus* α-toxin-induced cell death: predominant necrosis despite apoptotic caspase activation. *Cell Death and Differentiation* **10**:1260-1272. DOI: 10.1038/sj.cdd.4401301

Fei W, Hongjun Y, bin H, Changfa W, Yundon G, Qifeny Z, Xiaohong W and Yanjun Z (2011) Study on the hemolysin phenotype and genetype distribution of *Staphylococcus aureus* caused bovin mastitis in Shandong dairy farms. *Inter J Appl Res Vet Med* **9**: 416-421.

Gomha SM, Riyadh SM, Mahmmoud EA and Elaasser MM (2015) Synthesis and anticancer activities of thiazoles, 1,3-thiazines, and thiazolidine using chitosan-Grafted-Poly (Vinylpyridine) as basic Catalyst. *Heterocycles* **91**:1227-1243. DOI: 10.3987/COM-15-13210

Gurnev PA and Netorovich EM (2014) Channel-forming bacterial toxins in biosensing and macromolecule delivery toxins. *Toxins* **6**(8): 2483-2540. DOI: 10.3390/toxins6082483

Harlow E and Lane D (1988) *Antibodies, A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Haslinger B, Strangfeld K, Peters G, Schulze-Osthoff K and Sinha B (2003) *Staphylococcus aureus* α-toxin induces apoptosis in peripheral blood mononuclear cells: role of endogenous tumour necrosis factor- α and the mitochondrial death pathway. *Cellular Microbiology* **5**: 729-741. DOI: 10.1046/j.1462-5822.2003.00317.x

Inoue H, Nojima H and Okayama H (1990) High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96**: 23-28. DOI: 10.1016/0378-1119(90)90336-P

Johansson D, Johansson A and Behnam-Motlagh P (2008) α-toxin of *Staphylococcus aureus* overcomes a cquired cisplatin-resistance in malignant mesothelioma cells. *Cancer letter* **265**: 67-75. DOI: 10.1016/j.canlet.2008.02.007

Kong C, Neoh HM and Nathan S (2016) Targeting *Staphylococcus aureus* toxins: a potential from of anti-virulence therapy. *Toxins* **8**(3): 72 :DOI .10.3390/toxins8030072

Leng B-F, Qiu JZ, Dia XH, Dong J, Wang JF, Luo MJ, Li HE, Niu XD, Zhang Y, Ai YX and Dang XM (2011) Allicin reduces the production of α- toxin by *Staphylococcus aureus. Molecules* **16**(9): 7958-7968. DOI: 10.3390/molecules16097958

Liang X, Hall JW, Yang J, Yan M, Doll K, Bey R and Ji Y (2011) Identification of single nucleotide polymorphisms associated with hyperproduction of alpha-toxin in *Staphylococcus aureus.* PLoS ONE **6**(4): e18428. DOI: 10.1371/journal.pone.0018428

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. *J Immunol Methods* **65**: 55-63. DOI: 10.1016/0022-1759(83)90303-4

Ouyang P, Chen J, Sun M, Yin Z, Lin J, Fu H, Shu G, He C, Lv C, Deng X, Wang K, Geng Y and Yin L (2016) Imperatorin inhibits the expression of alpha- hemolysin in *Staphylococcus aureus* Strain BAA-1717 (USA300). *Antonie Van Leeuwenhoek* **109**(7): 915-922. DOI: 10.1007/s10482-016-0690-9

Pulicherla KK, Kumer A, Gadupudi GS, Korta SR and Rao KR (2013) In vitro characterization of a multifunctional Staphylokinase variant with reduced reocclusion, produced from salt inducible *E. coli* GJ1158. *Biomed Res Int* Article ID: 297305. DOI: 10.1155/2013/297305

Reddy YGP, Prakash R and Anandakumer S (2014) Isolation, cloning and expression of recombinant staphylokinase gene against thrombosis. *In J Pharm Pharm Sci* **6**(4): 266-270.

Rejendhran, J, and Gunasekaran, P (2010) Microbial phylogeny and diversity: small sub unit ribosomal RNA sequence analysis and beyond Microbiological research **166**: 99-110. DOI: 10.1016/j.micres.2010.02.003

Rubin, J E, Bayly, M K and Chirino-Jerjo, M (2010) Comparison of dog and rabbit plasmas in the tube coagulase test for *Staphylococcus aureus. J Vet Diagn Invest* **22**: 770. DOI: 10.1177/104063871002200521

Sogaard M, Norgaar M and Schonheyder HC (2007) First notification of positive blood cultures and the high accuracy of the gram stain report. *J Clin Microbiol* **45**: 1113. DOI: 10.1128/JCM.02523-06

Stulik L, Malafa S, Hudcova J, Rouha H, Henics BZ, Craven DE, Sonneren AM and Nagy E (2014) α-hemolysin activity of methicillin-susceptible *Staphylococcus aureus* predicts ventilator-associated pneumonia. *American Journal of Respiratory and Critical Care Medicine* **190**:1139. DOI: 10.1164/rccm.201406-1012OC

Swofford CA, Jean AT St Panteli JT, Brentzel ZJ and Forbes NS (2014) Identification of *Staphylococcus aureus* α-hemolysin as a protein drug that is secreted by anticancer bacteria and rapidly kills cancer cells. *Biotechnology and Bioengineering* **111**:1233. DOI: 10.1002/bit.25184

Tavares A, Nielsen JB, Boye K, Rohde S, Paulo AC, Westh H, Schonning K, Lencastre H and Miragaia M (2014) Insights into Alpha-hemolysin (*Hia*) Evolution and expression among *Staphylococcus aureus* clones with hospital and community 9 PLOS ONE

Vandenesch F, Lina G and Henry T (2012) *Staphylococcus aureus* hemolysin, Bio-component leukocidins, and cytolytic peptides: a redurdant arsenal of membrane-damaging virulence factors. *The Staphylococci and staphylococcal pathogenesis* 80

Wang JH, Niu HY, Zhang M, He P, Zhang Y and Kan L (2011) Apoptosis induced by *Sta*phylococcus aureus in human monocytic U937 cells involves Akt and mitogen-activated protein (MAPK) phosphorylation. African Journal of Biotechnology **10**(21): 4318-4327.