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RESEARCH ARTICLE

Immune Response Induced by Pure and Physical Detoxified *S.aureus* hemolysin Loaded on Calcium Phosphate Nanoparticles

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Abstract

A-hemolysin is one of the virulence factors that cause damage of RBC membranes. In the present study, *S. aureus* hemolysin was used as a model of toxin. Pathogenic strain of *S. aureus*, isolated from patient wounds, was used to produce α-hemolysin in tryptic soy broth medium. After 40 hrs of *S. aureus* growth, the supernatant was precipitated with 75% ammonium sulphate before purification by DEAE- cellulose and sephacryl-S200 chromatography. Detoxification of pure hemolysin by different temperature and two types of UV light had been achieved. Furthermore, calcium phosphate nanoparticles (CAP NPs) was prepared and used, as nanoadjuvant, to carry detoxified hemolysin. Scanning electron microscope (SEM), UV-VIS spectrophotometer, Zeta potential and X-ray diffraction (XRD) were used to characterize CAP and CAP/hemolysin complex. The CAP NPs appeared spherical in shape with diameter average 36-67nm, while, zeta potential of CAP NPs was -34.47 mV. The XRD patterns revealed the combination of CAP NPs with detoxified hemolysin and lost crystalline structure of CAP. Moreover, the combination was used to induce T-lymphocyte transformation and cytokines secretion in vitro. Lymphocyte proliferation with0.7 µg/ml of detoxified hemolysin increased proliferation 4.5 fold compared to negative control. On the other hand, 1.4 µg/ml of detoxified hemolysin stimulate the cytokines production of IL-1ß and IL-6 to 16.4 and 18 pg/ml, respectively.

Keywords: *Immune response, Detoxification, Hemolysin, Calcium phosphate, Nanoparticles.*

Introduction

S. aureus is the most pathogenic specie of the genus Staphylococcus, being implicated in both community-acquired and nosocomial infections. It often asymptomatically colonizes the skin and mucous membranes of healthy individuals, in particular the anterior nares [1]. In effect, it has been estimated that about 20-30 % of the population are permanently colonized by this bacterium, while other 30 % are transient carriers [2]. The colonization represents an increased risk of infection by providing a reservoir from which bacteria are introduced when the host defense is compromised [3]. Due to the importance of *S. aureus* infections and the increasing prevalence of antibiotic-resistant strains, this bacterium has become the most studied staphylococcal species [4]. A-hemolysins are extracellular toxic proteins which are produced by many gram negative (e.g. *Escherichia coli*, *Serratia spp*., *Proteus spp*., *Vibrio spp*., *Pasteurella spp*., *Pseudomonas aeruginosa*) and gram positive bacteria (e.g. *Streptococcus spp*., *Staphylococcus aureus*, *Listeria spp*., *Bacillus cerius*, *Clostridium tetani*), all of which possess a certain pathogenic potential. A-hemolysins have been therefore always considered as virulence factors. α-hemolysins cause lysis of erythrocytes by forming pores of varying diameters in the membrane and are designated as such because they have the ability to lyse red blood cells (RBCs).Many hemolysins can also attack –probably by a similar mechanism-other mammalian cells. Due to this cytolytic effect, they are also termed cytolysins [5].Vaccination to protect against human infectious diseases may be enhanced by using adjuvants that can selectively stimulate immunoregulatory

responses. Calcium phosphate (CA) is a constituent of the human body was more potent to use as adjuvant with the absence of side effects[6].A-hemolysin might be a stronger inducer of the inflammasome to be an important intracellular signaling complex [7].Pro-inflammatory cytokines are often present at elevated levels in the course of inflammatory responses and infections. Particularly, the prototypic pro-inflammatory cytokine IL-1ß might have a direct impact on CD^4 T cell expansion. The IL-1 effect was action on antigen presenting cells (APCs) and its effect depended on the expression of the responding T cells, implying that it may have mimicked the action of TLR-engaging or inflammasome-activating.

Indeed it had been shown that IL-1 plays a role in the regulation of DC activation , enabling the production of cytokines and enhancing the differentiation of T cells [8 ,9].The aims of present study were to produce α- hemolysin from *S. aureus* and detoxified by different temperature and two types of UV (A and C). The detoxified toxin was loaded by calcium phosphate nanoparticles (CAP NPs) after characterized by spectrophotometer, Zeta potential, SEM and XRD. The immune response was determined *in vitro* when detoxified hemolysin was used after combined CP NPs.

Materials and Methods

Production of Hemolysin

Tryptone soya broth medium was used to produce hemolysin [10]. A 50 ml of *S. aureus* suspension (10^6 cells /ml) was inoculate in 1000 mL of production medium and incubated at 37 °C for 40 hrs.

Hemolysin Activity Assay

Hemolytic activity of α-hemolysin was determined according to the procedure that mentioned in [11] with some modification. In brief, 200 µL of the separated blood was mixed with 1600 µL of normal saline. Then, 200 µL of different concentration of supernatant (5, 10, 20, 40 and 80 µL mL^{-1}) were added to the diluted blood .The samples were incubated at 37°C for 1 h and centrifuged at 700 rpm for 5 min. Positive and negative control include DW and normal saline, respectively, were used for comparison. Samples absorbance was measured spectrophotometry at 541 nm and hemolysis percentage was estimated by using the following formula:

$$\text{Hemolysis (\%)} = \frac{\text{Abs. of sample} - \text{Abs. of negative control}}{\text{Abs. of positive control} - \text{Abs. of negative control}} \times 100$$

Purification of Hemolysin

The culture supernatant was fractionated by ammonium sulphate at 75 % saturation. Concentrated hemolysin (10 ml) was passed onto DEAE cellulose column (3x 10 cm) gradually. Elution was carried out with 10 mM sodium acetate buffer (pH 5) containing 200 mM NaCl. Moreover, Sephacryl S-200 column (50 x1.5 cm) was used for more purification. The column was equilibrated with 10mM sodium acetate buffer (pH 5) containing 20mM NaCl [12]. Fractions of 5ml were collected and the presence of the protein and hemolysis activity was estimated spectrophotometry in each fraction.

Physical Detoxification of Hemolysin

Detoxification of α-hemolysin by different temperature and two types of UV light had been applied. Pure hemolysin was incubated in different temperatures (30, 40, 50, 60 and 70 ^{0}C) for 30 and 60 min, separately. On the other hand, ultra-violate radiation type A and C were used to detoxify pure hemolysin , distributed in plates , with exposing time of 5, 10, 20 and 40 min and the distance was 5 cm from the UV source. Activity of the hemolysin was measure as described previously.

Loaded Detoxify Hemolysin on Calcium Phosphate NPs

CAP NPs were synthesized by reacting12.5 mM calcium chloride, 12.5 mM dibasic sodium phosphate, and 15.6 mM sodium citrate were mixed together and stirred for 48hr, after that sonication for 30min. Detoxified hemolysin was loaded according to the procedure that mentioned [6]. Protein was added to 12.5mM calcium chloride, followed by the addition of 12.5 mM dibasic sodium phosphate and 15.6

mM sodium citrate. The solution was stirred overnight at 4ºC. CAPNPs was characterize alone or in the presence of detoxified hemolysin.

Characterization

Spectrophotometer

The UV analysis was carried out by scanning the prepared solution of CAP NPs by UV/Vis spectrophotometer in range of 200-1000 nm.

Scanning Electron Microscope (SEM)

SEM analysis was done by dispersing 10 µl of CAP NPs on cover slide and dried at room temperature.

Zeta Potential

The zeta potential analysis of prepared CAP NPs was determined. The solution potential was measured by electrode range from -160 to 160 mv.

X-Ray Diffraction

XRD pattern was determined for precipitated CAP NPs on 1 cm^2 glass slide at 40 KV operating voltage and 30 mA current with Cu radiation and scan range of 10.0000 - 80.0000 (deg).

Lymphocyte Transformation Test

Five milliliters of whole blood transferred into tubes, contained lithium heparin, 250 µl of whole blood mixed gently with 0.7 or 1.4 µg/ml detoxified hemolysin and incubated at 37 °C for 72 hrs. Thereafter, tubes were centrifuged at 3000 rpm for 5 min. The sediment was transferred into sterile tubes and 5 ml of hypotonic solution was added and incubated again at 37 °C for 50 min. The centrifugation step was repeated again and 5ml of fixation solution (3:1 absolute ethanol and acetic acid) was added and incubated at 4°C for 15 min. Fixation step were repeated even get clear colorless precipitation, then suspended in 0.5ml of the fixation solution.

Then, the suspension was dropped on glass slide and left to dray in room temperature. Thereafter, stained with Giemsa stain for 15 minutes before washing and dried again. Negative control, includes 2.5 ml of RPMI-1640 medium and 250 µl of whole blood, and positive control, includes Phytohaemagglutinin (PHA) 5 µg / ml more than negative control, were used. Lymphocytes (including lymphoblast and non-dividing lymphocytes) were counted under light microscope according to the following equation [14]:

$$\text{Transformed cells (\%)} = \frac{\text{Lymphoblast number}}{\text{Total number (lymphoblast + lymphocyte)}} * 100$$

Determination of the Cytokine Level by ELISA Technique

Venous whole blood sample was collected from healthy person never took medicines for at least 10 days before. In order to determine the cytokines level, phosphate buffer saline (pH 7.2) was added to the 5 ml whole blood, mixed previously with 0.2 % EDTA, in ratio of 1:1. The whole mixture was added carefully to the Ficoll separation fluid (lymphprep; specific gravity=1.077g/l) in ratio 2:1.

The sample was centrifuged at 2000 rpm for 30 min. The lymphocyte layer was collected by Pasteur pipette and then the suspension placed into 10 ml tube. Wash the lymphocyte twice with 5ml RPMI-1640 media, centrifuged for 10min at 2000 rpm. The lymphocytes were re-suspended in RPMI-1640 media for further investigation [15].

Using tissue culture plate, 0.1, 0.3, 0.7 and 1.4 µg/ml of detoxified hemolysin was added 200 µl of lymphocyte suspension.

Plates were incubated at least for 4 hours at 37°C, then harvested into appendorf tubes centrifuged for 20min at 2000 rpm. This process above was repeated at least three times to increase cytokine amount. With the final centrifugation supernatant containing cytokine were taken and freeze at -20°C for further study and measuring IL-1ß and IL-6 using ELISA [16].

Result and Discussion

Production and Purification of A-hemolysin

S. aureus was incubated in tryptic soya broth (TSB) at 37 ºC. Hemolysis activity of α-

hemolysin was determined during incubation, as presented in figure (1). The results showed that the α-hemolysin production was increased with time and hemolysis activity was higher at 40 hrs. Kong and his colleagues produced α- hemolysin toxin from *S.aureus* and induced toxin production in TSB medium [10].

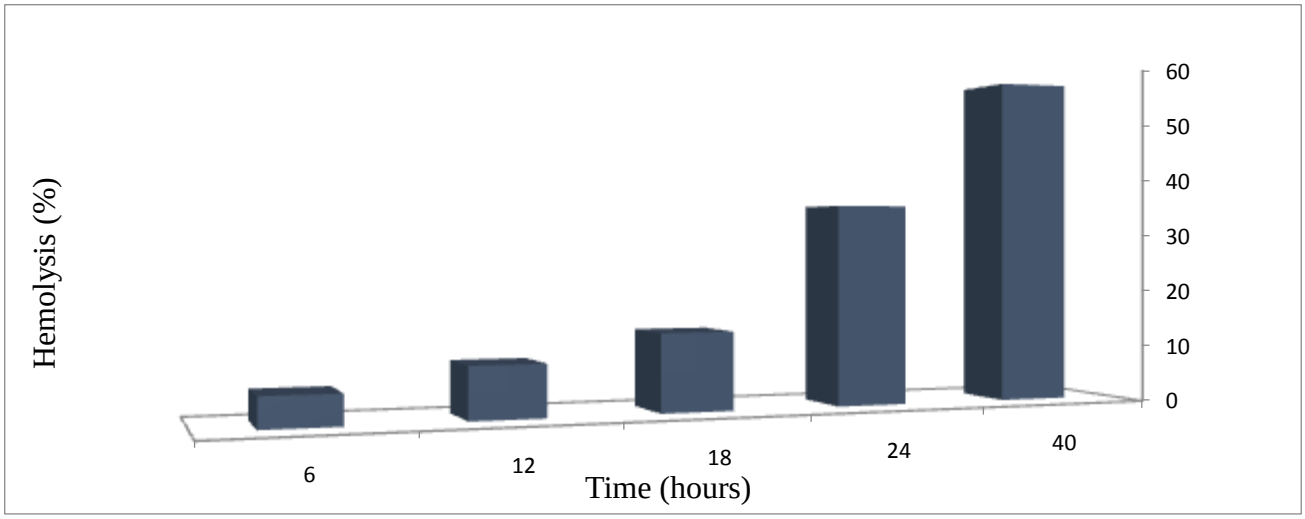

Figure 1: α- hemolysin production by *S. aureus* in TSB medium at 37 °C

Hemolysin was precipitated in 75 % saturations of ammonium sulfate in order to concentrate the crude toxin to 15ml, hemolysis activity (52.3 %) and protein concentration (0.9 mg/ml) of crude toxin were obtained and final volume 10 ml after dialyzed overnight. Lind and his colleagues found that ammonium sulfate, with 75% saturation, is the highest ratio to precipitate α-hemolysin [17]. This step allows the salting out of molecules from water. Since ammonium sulfate has the ability to neutralize charges at the surface of the protein and to disrupt water layer surrounds the protein and eventually cause decreasing in the solubility of protein which, in turn lead to the precipitation of the protein [18, 19]**.** DEAE-Cellulose ion exchanger was used to purify 10 ml of crude hemolysin obtained from salt fractionation. Hemolysin was eluted from column by 200 mM NaCl in 10 mM sodium acetate (pH 5). The absorbance of the washing parts was measured at 280nm after restoring the protein process associated with the ion exchanger was performed. The results of the ionosphere chromatography analysis showed that absorbance at 280nm revealed the protein existence and hemolysis activity in tubes (21-27), while negative tubes were discarded, as shown in Figure (2). The positive tubes were mixed (35 ml) and concentrated in sucrose to 10 ml. Activity reached (35%) and the protein concentration (0.490mg/ml) and final volume 10 ml.

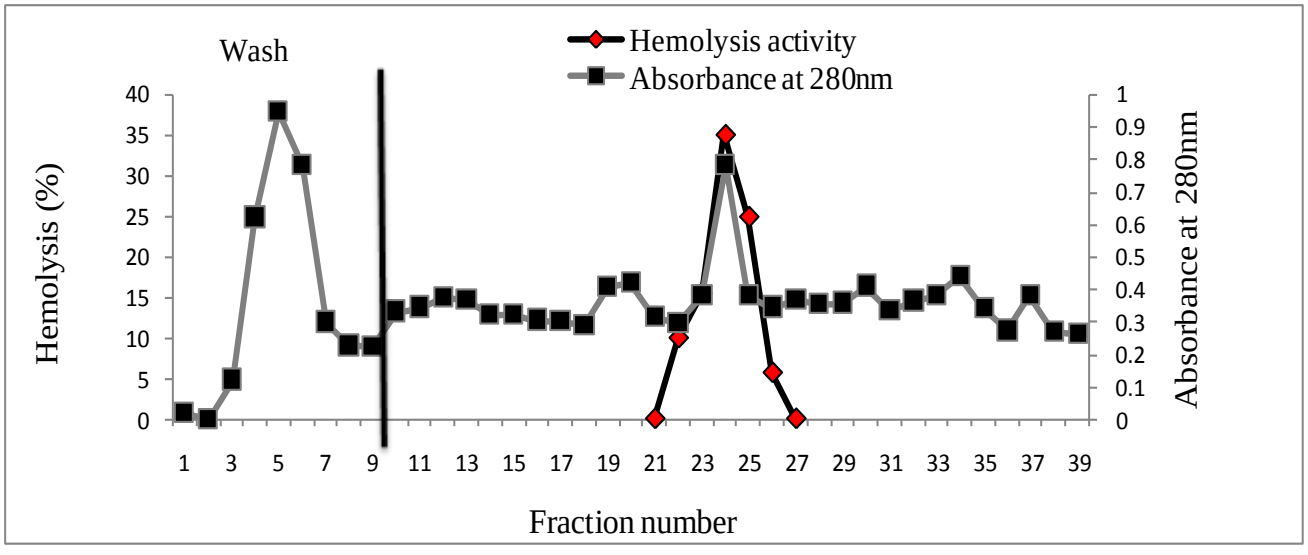

Figure 2: Purification of *S.aureus* α-hemolysin in DEAE-cellulose column (3 x10 cm), equilibrated with 10mM acetate buffer pH 5.0, flow rate 60 ml/hr and fraction volume 5ml

Purification carried out by gel filtration using Sephacryl S-200. Hemolysin obtained from DEAE cellulose column (10 ml) was passed through gel filtration column (1.5 x50 cm). One peak of absorbance at 280nm was appeared in fractions 7-16. The protein activity was 17.5%, as shown in Figure (3), while protein concentration was (0.066mg/ml). The tubes were collected and concentrated in sucrose to 3 ml.

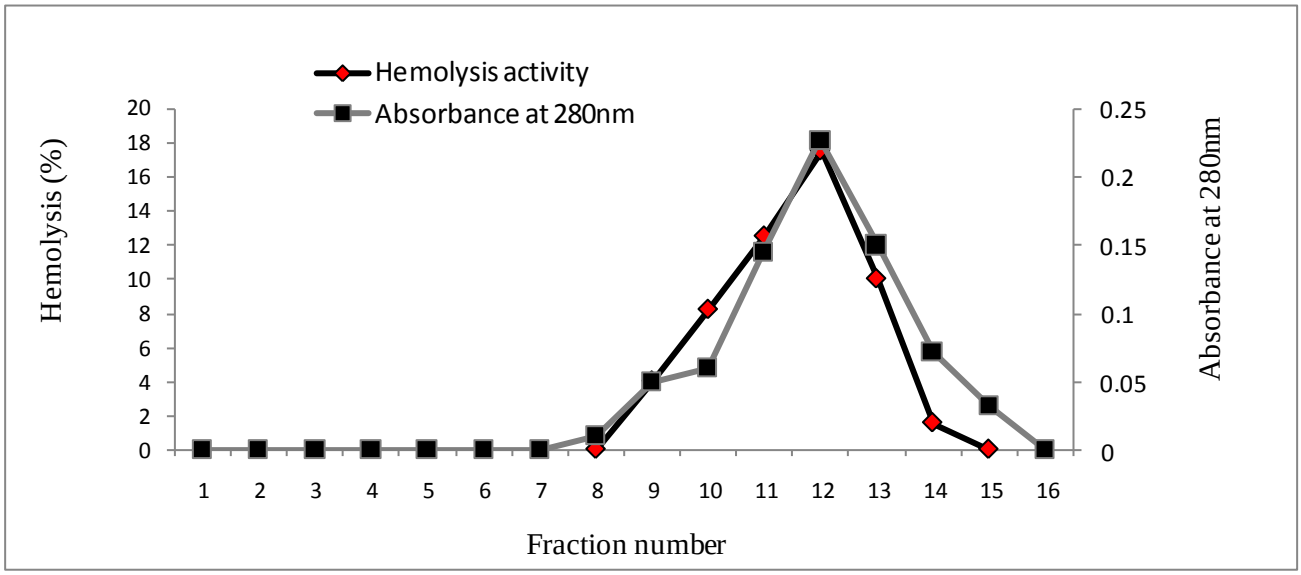

Figure 3: Purification of *S. aureus* α-hemolysin in Sephacryl S-200 column (1.5 x50 cm), equilibrated with 10mM acetate buffer pH 5.0, flow rate 60 ml/hr and fraction volume 5ml

Physical Detoxification of A-hemolysin

Different incubation temperatures were used to reduce the activity of purified α-hemolysin within 30 or 60 min, as presented in figure (4). The results showed that the activity of toxin reached its maximum activity at 30 and 40 ºC .The hemolysis activity then decreased whenever temperature exceed more than 40 °C , and the detoxification was observed at 70 °C within 60 min . A-hemolysin maintains its hemolytic activity with a wide range of temperature related to the type of organism. The effect of temperature on the purified hemolysin activity was due to a change in the nature of the protein structures resulting from the breakdown of ionic bond and the weakness of hydrogen bond that stabilize the three-dimensional structure of the protein [20]. Furthermore, protein denaturation is often irreversible because of covalent bond breaking and/or aggregation of unfolded protein. Temperature variations lead to changes in both the volume and the thermal energy of protein and it is difficult to separate these effects [21]. It is agreed that most proteins are more stable in low temperature, and the most active and optimal temperature for α-hemolysin was 35 ºC [22].

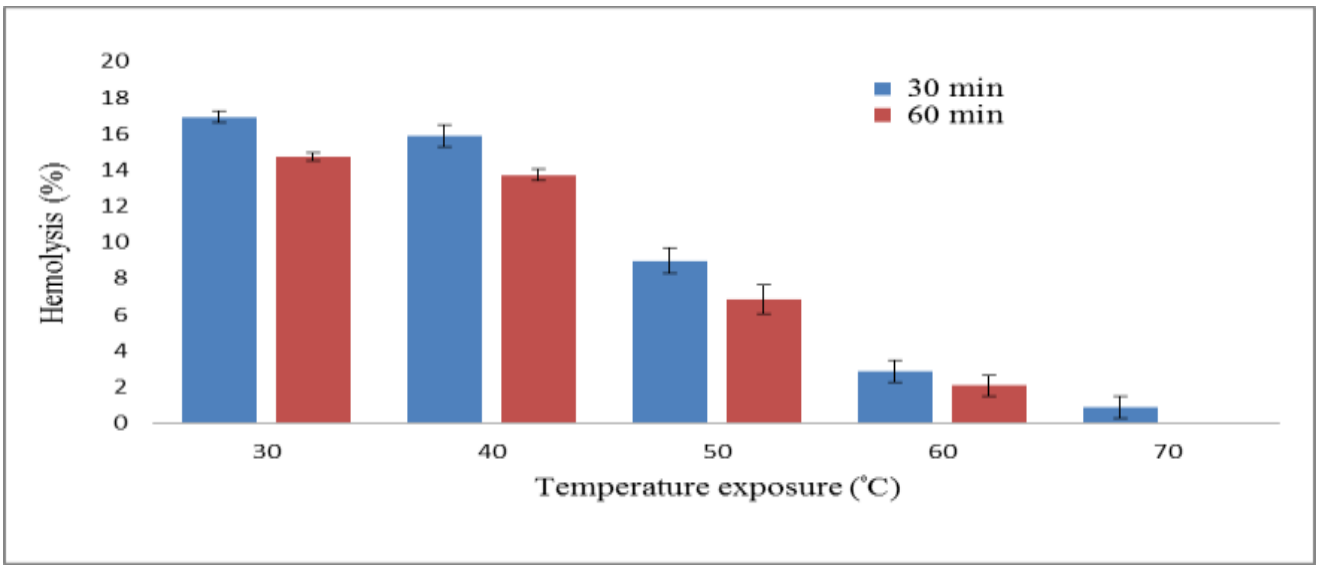

Figure 4: Detoxification of *S. aureus* α-hemolysin by different temperature with time

On the other hand, the exposing of α-hemolysin to UVA and UVC radiation under different durations was investigated. The results in figure (5) showed that the activity of alpha-hemolysis was reduced to 13.5 and 11.65 % after 5 min of exposure to UVA and UVC, respectively. The detoxification of toxin was clearly observed when the toxin exposure to the UVA and UVC within 40 min.

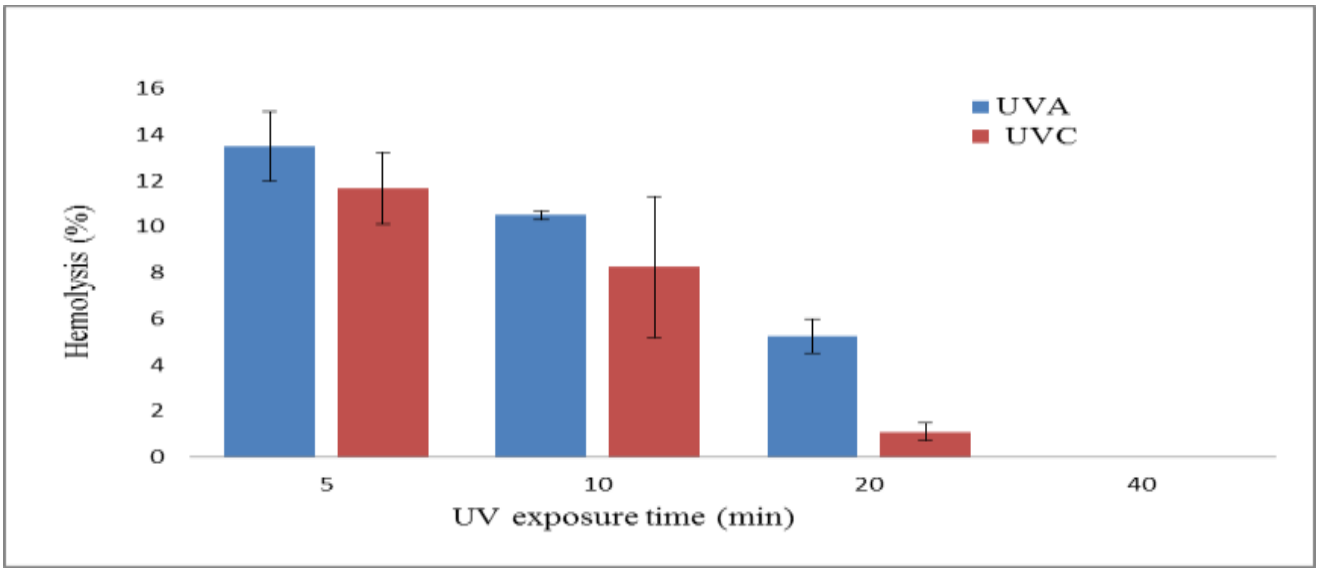

Figure 5: Detoxification of *S. aureus* α-hemolysin by UVA and UVC with time

Ultraviolet ray is a type of electromagnetic (EM) radiation led to damage in the functional structure of proteins, inactivation and causes some diseases. Electron transfers to the nearby disulfide bonds formed between cysteine amino acids when the protein absorbed the UV. UVA radiation has longest wavelengths (315–400 nm), while UVC radiation has shortest wavelengths (180–280 nm) [23, 24]. UVC region as the most biologically effective wavelength, identified for DNA and protein as the principal damage target. As a consequence, most research into the biological effects of UV has been based on lamps emitting predominantly UVC radiation. While, UVA region were generating various reactive oxygen species (ROS) Moreover, most UVA-mediated biological events are oxygen-dependent. These observations provided the basis for considering UVA as a strong generator of oxidative stress [25].

Characterization of CAP NPs

CAP-NPs absorbance was measured using UV-VIS scanner spectrophotometry ranged from 200-1000nm wavelengths. The result in figure (6) showed the scan of CAP NPs spectra with detected absorbance at 276nm. The results are extremely agreed with the results that referred to the detection of CAP NPs in 276nm [26].Another study showed that CAP-NPs was could be synthesized by the chemical method can be detected at absorbance between 230–260 nm [27].

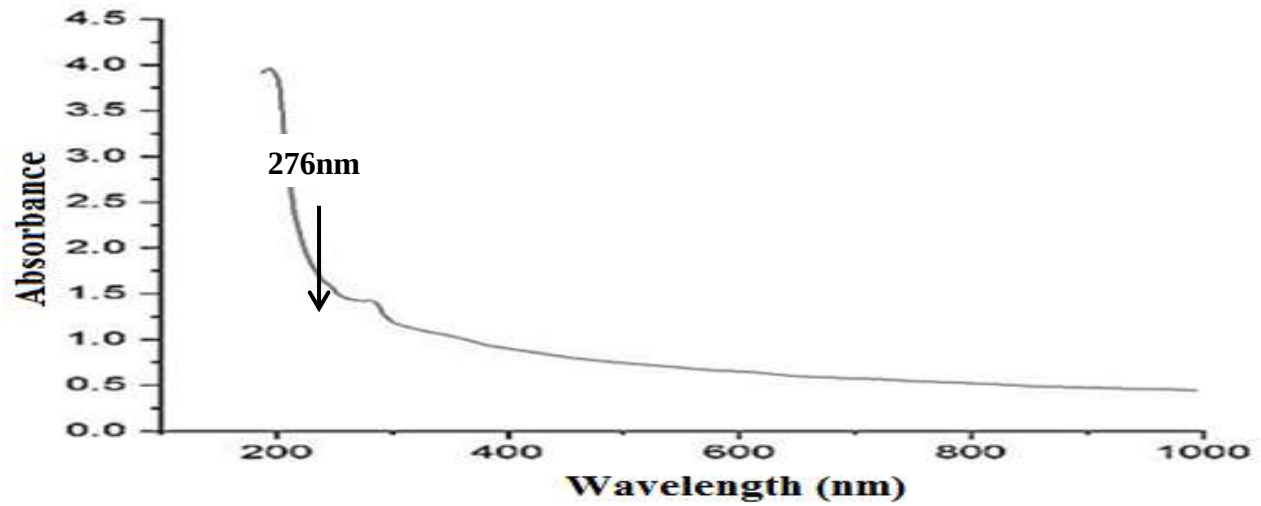

Figure 6: Spectra scan of CAP-NPs

Moreover, SEM technique was employed to measure the size and visualize the shape of CAP-NPs. SEM revealed that CAP-NPs are spherical particles with diameter ranged from36 to 67nm [28], as presented in Figure (7).

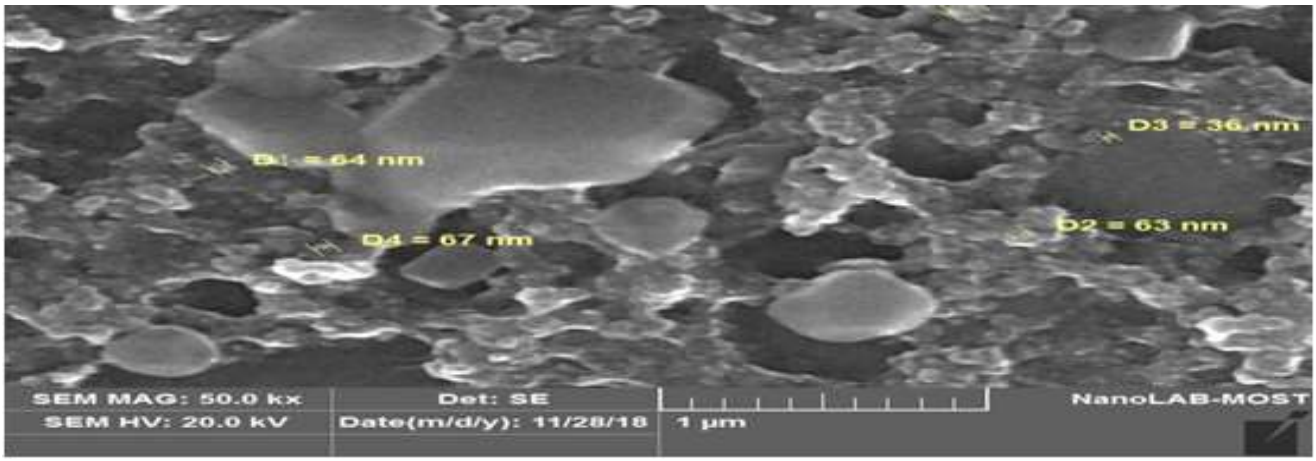

Figure 7: SEM image of CAP-NPs

On the other hand, zeta potential is widely used for quantification of the magnitude of the electrical charge. The average zeta potential of CAP-NPs was found to be -34.47 mV as shown in Figure (8). Stable CAP-NPs, from -30 to +30, prevent flocculation and aggregation of the nanoparticles [29].

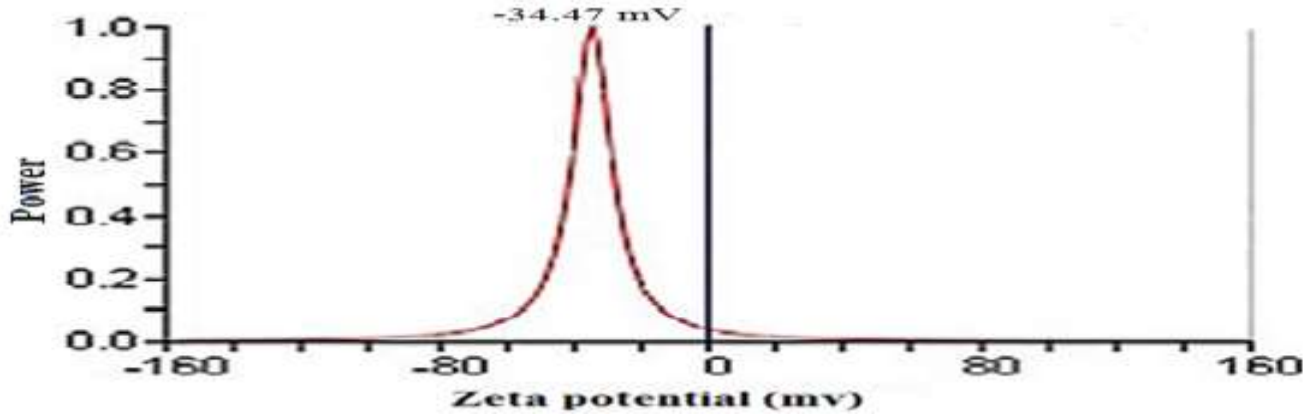

Figure 8: Determination of CAP-NPs zeta potential

Furthermore, XRD patterns of both CAP-NPs and CAP- NPs with detoxified hemolysin are represented in Figure (9). Crystalline structure of free CAP-NPs distanced by diffraction peaks at 25, 31and 41degrees. The pattern of CAP-NPs with hemolysin revealed disappearing in peaks at 30 and 40 degrees, due to the diffuse and binding NPs with hemolysin.

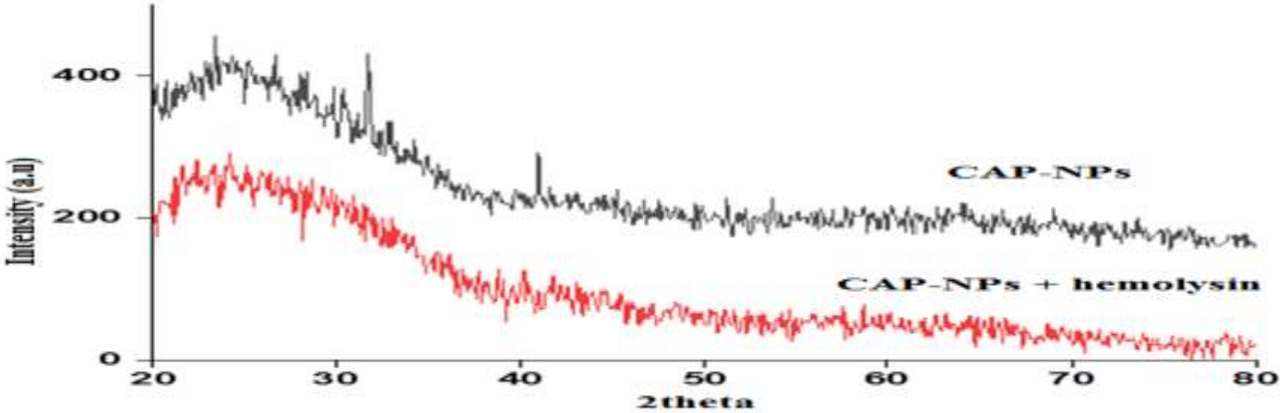

Figure 10: XRD patterns of single CAP- NPs or in combination with hemolysin

Lymphocyte Transformation and Cytokines Detection

Lymphocyte stimulation was induced by different concentrations of detoxified hemolysin, as showed in Figure (11). Detoxified hemolysin at a concentration of 1.4µg/ml showed higher lymphocyte proliferation and six times higher than negative control. Lymphocyte incubated with (PHA) alone for 72 hrs substantially promote proliferation with 3-fold increasing, while 0.7µg/ml of detoxified hemolysin increased 4.5-fold when compared to negative control. Figure (12) presented the lymphocyte proliferation after 72 hrs of incubation at 37 °C.

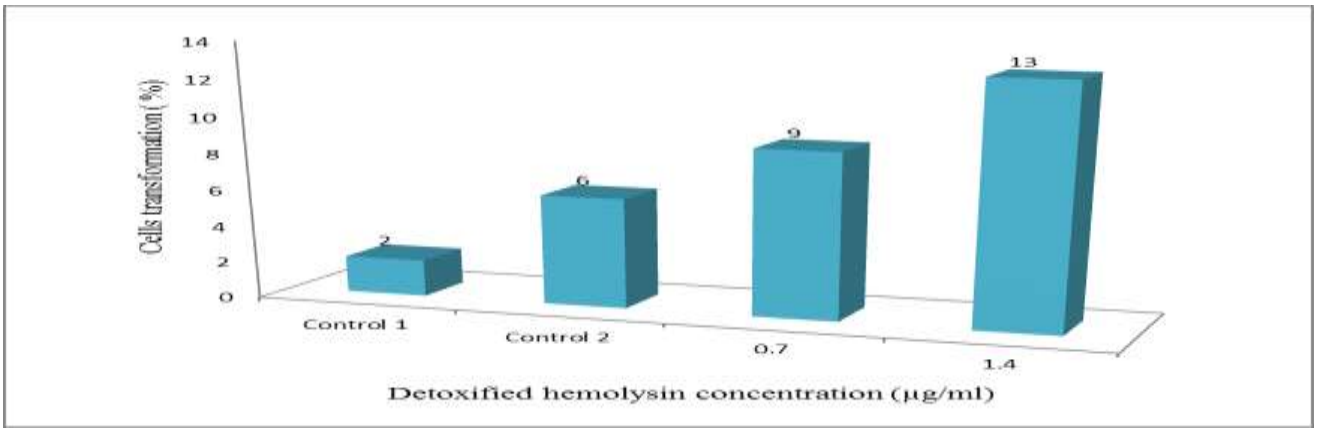

Figure 11: **Lymphocyte transformation (%) after incubated with (PHA) and detoxified hemolysin for 72 hrs. Control 1: without detoxified hemolysin and control 2: PHA only (5 µg/ml)**

The biomimetic nanoparticle system offers a versatile approach for vaccine development against many infectious diseases [30]. In present study, nanoparticles have demonstrated unique advantages in enhancing immunotherapy potency interest in developing safe and effective vaccine formulation. Vaccination against virulence factors through detoxified bacterial toxin sequestration and loaded on nanoparticles can be directly target APCs with both T and B cell epitopes, which induce both cellular and humoral immune responses essential for treatment of various infections [31]. Detoxified hemolysin was exhibit various pathogen associated-molecular patterns (PAMP) that play a key role in stimulating innate immunity and promoting adaptive immune responses [32]. $CD^{+4}T$ cells play a critical role in mediating adaptive immunity to a variety of pathogens. The detoxified hemolysin stimulates lymphocytes to proliferate and differentiate. Niebuhf and his colleagues found that the hemolysin induce lymphocyte proliferation [33].

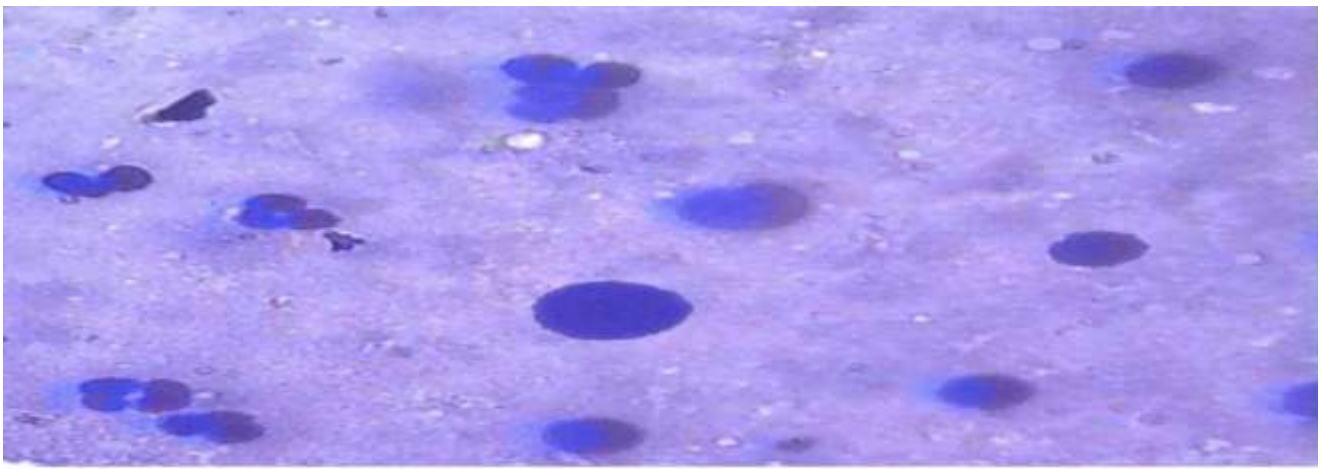

Figure 12: Lymphocyte proliferation after 72 hrs of incubation at 37 °C with detoxified hemolysin

On the other hand, detoxified hemolysin was responsible for activation of inflammatory responses include maturation and secretion of inflammatory cytokines (IL-1ß and IL6). The results showed that 0.1 and 0.3 µg/ml of detoxified hemolysin did not stimulate cytokines production. On the other hand, 0.7 and 1.4µg/ml of detoxified hemolysin,

stimulate the production of cytokine IL-1ß to 2.8 and 16.4 pg/ml and cytokine IL-6 to 1 and 18 pg /ml, respectively, as shown in Figure (13).

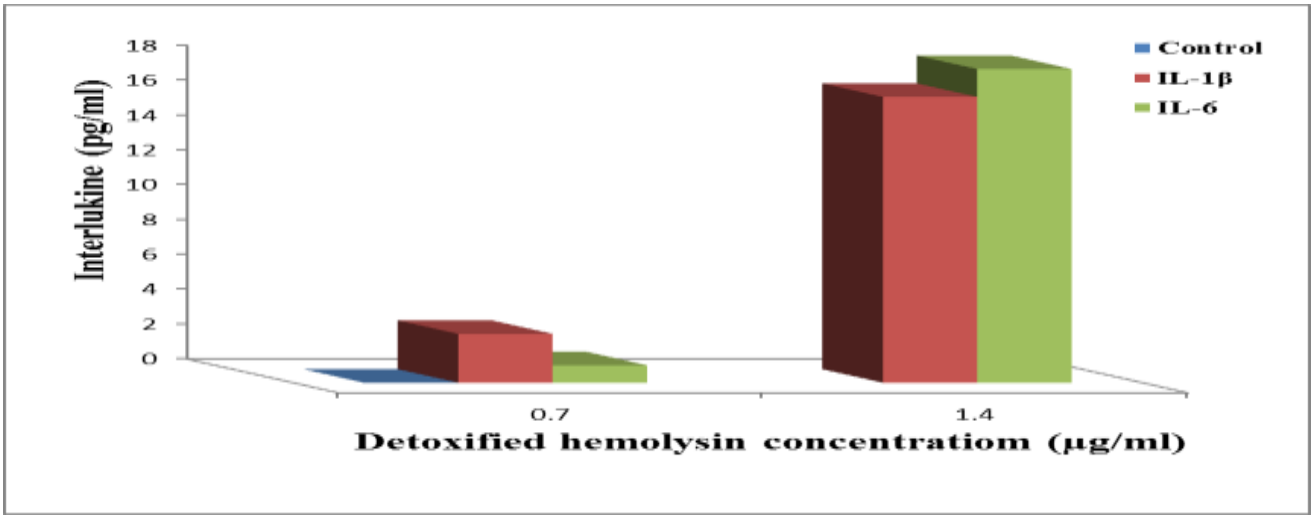

Figure 13: Detoxified hemolysin -induced IL-1β and IL-6 production determined by ELISA assay

α-hemolysin has ability to induce inflammatory signaling and activation of caspase-1 through the nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome, and could be inducing IL-1ß and IL- 6 secretion [34,35]. Inflammasomes are multiprotein complexes found in monocytic-lineage cells. They are classified in part by specific cytosolic pathogen recognition receptors (PRR) that, in response to danger signals, assemble to activate caspase-1.

The PRR (NLRP3) is known to respond to stimuli, including efflux of potassium (K+) that can be induced by pore-forming toxins. NLRP3 then can activate caspase-1 via the ASC (apoptosis-associated speck-like protein containing a CARD domain) adaptor protein. Active caspase-1 cleaves and activates proinflammatory cytokines IL-1ß, leading to their secretion, these cytokines, in turn, recruit monocytes and macrophages to the site of infection and induces Th1 and Th17 adaptive immunity [36, 37]. Inflammation is the immune system's response to harmful stimuli, such as pathogens, damaged cells or toxic compounds, initiates a chemical signaling cascade that stimulates responses aimed at healing affected.

These signals activate leukocyte chemotaxis from the general circulation to sites of damage. These activated leukocytes produce cytokines that induce inflammatory responses and acts by removing injurious stimuli and initiating the healing process. Inflammation is therefore a defense mechanism that is vital to health [38].

The principal functions of the immune system are the recognition and elimination of foreign antigens, formation of immunologic memory.

T lymphocytes and B lymphocytes which work in close collaboration with the innate immune system $CD^{+4}T$ cells along with $CD^{+8}T$ cells make up the majority of T-lymphocytes. $CD^{+4}T$ cells after being activated and differentiated into distinct effector subtypes play a major role in mediating immune response through the secretion of specific cytokines [39].

Inactivated vaccines are presented in the context of second-class MHC molecules to CD^{+4} T lymphocytes. Vaccination with live attenuated vaccines can lead to the intracellular production of antigenic peptides within antigen-presenting cells, which are then presented to CD^{+8} T lymphocytes in the context of MHC first class molecules. T cells recognize the MHC/antigen complex with their specific T cell receptors. This leads to T cell activation, clonal expansion of effector T cells, and the formation of long-lived memory T cells, the hallmark of adaptive immunity. In the case of primary exposure to an antigen, naive, antigen-inexperienced T cells are activated. On booster vaccination, preexisting memory T cells recognize antigen-loaded dendritic cells, expand rapidly, and differentiate into effector T cells, which leads to a faster and stronger memory response[40].

Conclusion

Separation and purification of α-hemolysin using ion exchange and gel filtration

chromatography provide simple and rapid technique for protein purification and the molecular weight of purified α-hemolysin estimated as 36.545 KD. Removal of hemolysin toxicity can be achieved physically using heat and radiation. The use of CAP NPs to deliver α-hemolysin into cells is possible. Detoxified α-hemolysin can induce immune cells proliferation and cytokines production.

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