








Inhibition of *Staphylococcus aureus* enterotoxin genes by using plant extracts

Inhibición de genes de enterotoxinas de *Staphylococcus aureus* usando extracto de plantas

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ABSTRACT

Introduction: Enterotoxigenic *Staphylococcus aureus* is an important pathogen that causes septicemia and bacteremia and is often associated with serious complications, such as endocarditis and osteomyelitis. Some *Staphylococcus* enterotoxins require only minute quantities to be toxic in humans. The present study focused on investigation how to remove this problematic issue. **Objectives:** This study was conducted to inhibit *S. aureus* enterotoxin genes that obtained from positive blood culture bottles of patients at the pediatric hospital in Sulaimania city. **Methods:** Twenty five isolates of *S. aureus* were isolated among 100 positive blood culture bottles and determined the strains that produce enterotoxins through culture method. Then, the enterotoxin genes that located on plasmids were cured by two medicinal plants (*Eugenia caryophyllata* and *Cinnamomum zeylanicum*). **Results:** The results showed that nine out of 25 isolates were released enterotoxins from which the plasmid encoding enterotoxin genes were confirmed in four of them. And, two of the isolates were transferred to recipient DH10B *E. coli* isolate successfully. Methanol extracts of (*E. caryophyllata* and *C. zeylanicum*) were used at sub minimum inhibition concentration as curing agents. **Conclusion:** Methanol extracts of (*E. caryophyllata* and *C. zeylanicum*) have grate effect on eliminating the plasmids encoding enterotoxin gene of *S. aureus*.

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INTRODUCTION

Staphylococcus aureus is an important pathogen. The bacterium is known as one of the most frequent pathogens in both community and nosocomial infections, and it causes septicemia, endocarditis, osteomyelitis, abscesses, pneumonia, wound infections, impetigo, cutaneous rash, in addition to various toxin-mediated disease (1). In addition the combinations of toxin-mediated virulence, invasiveness, and antibiotic resistance are also play a major role in these manifestations (2). Blood stream infection is often associated with serious complications. The complication rates rise with duration of untreated bacteremia (3). *S. aureus* is one of the leading causes of bacteremia, with high levels of accompanying morbidity and mortality (4). Furthermore, microbial illnesses are regulated by an array of virulence factors that contribute to the pathophysiology and survival of the pathogen in the host (5). These factors include cell surface proteins, receptors, and adhesins that facilitate host colonization; capsular polysaccharides that confer protection from host immune system, and microbial toxins that cause host tissue damage (6,7). Since microbial toxins play a prime role in the virulence and pathogenesis of microbes in the host, they are major targets for developing plant-derived compounds such as therapeutic interventions (8). Reducing the expression of these virulence factors could control infections in humans (9). Current study, focused on the effects of *Eugenia caryophyllata* and *Cinnamomum zeylanicum* crude extracts as antimicrobials agents against enterotoxin gene of *S. aureus*.

MATERIALS AND METHOD

Isolation and Identification: (100) samples of blood culture bottles were obtained directly from laboratory of Sulaimania Pediatric Hospital which have obtained from high fever patients with suspect-

ed sepsis or bacteremia. Specimens were collected using the BacT/Alert bottles which must be accessioned and processed as quickly as possible. The computer detects a positive results by an increased rate of colour change to turbid (2). Then, (0.01ml) of the suspected blood samples were streaked onto blood agar and incubated at 37°C for overnight. The presumptive colonies of *S. aureus* were further cultured onto mannitol salt agar (MSA) and repeatedly sub-cultured to get pure culture. The isolates were identified as *S. aureus* on the basis of Gram staining, colony morphology (10), and biochemical tests (catalase test, coagulase test, DNase test and gelatinase test) (11).

Production of Enterotoxins: Purified bacterial colony were stabbed into brain heart infusion slant, after incubation for 24 hours at 37 °C, one loopful of the growth from the slant was transferred to 5 ml tubes of sterile saline solution to obtain a McFarland turbidity value of 300 CFU(x 10⁹). Four drops of this bacterial suspension were spread over the surface of brain heart infusion agar plate supplemented with phenol red (0.02gm/100ml) (pH 5.4), and then the medium was incubated at 37°C for 24 hours. A positive result (enterotoxin production) was indicated by the medium color from yellow to red due to the change of phenol red color as respond to pH changes (from 5.4 to 8.2) (12).

Isolation of plasmid DNA: Plasmid extractions were performed essentially by alkaline lysis method. A single colony of bacterial isolates was grown in 10 ml of LB broth containing 50µg/ml penicillin G and incubated at 37°C for 24 hr. with shaking. Bacterial cells were harvested by centrifugation at 10000 rpm for 10min, and then transferred to sterile eppendorf tube when plasmid samples were turbid, proteinase K was added to the samples to a final concentration of 50 µg/ml and the mixtures were incubated

for 30 min at 37°C. After incubation with proteinase K, plasmid DNA was extracted with phenol-chloroform and then precipitated with ethanol (13).

Transformation processes include:

1-Competent cells preparation: Many bacteria can be made competent by exposure to a divalent or multivalent cation, such as calcium chloride, manganese chloride (14).

2- DNA uptake: 100µl of prepared plasmid DNA (0.1) ml added to tube containing 0.2 ml of competent cells. The mixture was placed on ice for 30 minutes, exposed to heat shock at 42 °C for 6 minutes (15). After that 1ml of fresh nutrient broth was added to transformation mixture, and incubated at 37C° for 60 minutes to allow the expression of the enterotoxin genes. All samples of 0.1 ml from transformation mixture were spread on the plates of brain heart infusion agar supplemented with phenol red (pH 5.4) by adding (0.02gm/100ml) (16), and 0.1 ml of competent cells spread on brain heart infusion agar containing phenol red as control. All plates were incubated at 37 °C for 24 hours for the selection of transformants in DH10B *E. coli* as described by (17).

Electrophoresis of plasmid DNA: Plasmid DNA was subjected to electrophoresis at 100 mA for 1.5 h, using horizontal 0.8% agarose gels in TAE buffer (pH 8.0) containing 50 mM Tris, 20 mM sodium acetate, 2 mM EDTA, and 18 mM NaCl (18). Gels were stained with ethidium bromide (2µl).

Selection of medical plants for study: Clove (*Eugenia caryophyllata* and *Cinnamomum zeylanicum*) were obtained from local market in Sulaimani city then Preparation of methanol crude extracts were performed (19).

Determination of minimum inhibitory concentration (MIC): The minimum inhibitory concentration was determined for plant extract, which inhibited bacterial growth. The test was compared to the control sample which consisted of 5 ml of nutrient broth and 0.1 ml of overnight culture of bacterial suspension, and then incubated at 37°C for 24 hours. The MIC of medicinal plant extracts were determined by turbidity method (spectrophotometric method) at 600 nm and the following dilution were prepared for each extract (100, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000 and 7000) µg/ml (20). In addition, the sub minimum inhibition concentration (SMIC) of medicinal plant extracts was determined and used as curing agents.

RESULTS

Isolation and Identification of *Staphylococcus aureus*

25 isolates of *S. aureus* from 100 samples of blood culture bottles were isolated and identified by fermenting the sugar of mannitol and convert the pink color of the mannitol agar to (yellow) color, on blood agar which appeared as a smooth colony yellow or golden in color, some of the strains produce beta hemolytic or clear zone around the colonies. Morphologically as gram positive cocci arranged in grape-like irregular clusters (21,22).

Biochemical activity

S. aureus positive for catalase, coagulase, DNase activity and gelatinase or liquefaction of gelatin (23,21). Thus the results showed 25 isolates of *S. aureus* were isolated among 100 positive blood culture bottles or samples.

Isolation of Enterotoxigenic *S. aureus*

Twenty five isolates of *S. aureus* that isolated from positive blood culture bottles nine of them were ob-

served as enterotoxin producer by cultural method as shown in figure (1). The result showed in figure (1- a) the yellow brain heart infusion agar (BHIA) with special indicator without inoculation with bacterial strain and used as a control. Figure (1-b) showed a

positive result (enterotoxin production) which was indicated by a change in the medium color from yellow to red. This was due to the change of phenol red color as respond to pH changes from (5.4 to 8.2) for isolates no. (11, 23, 38, 41, 67, 81, 88, 89, and 95)

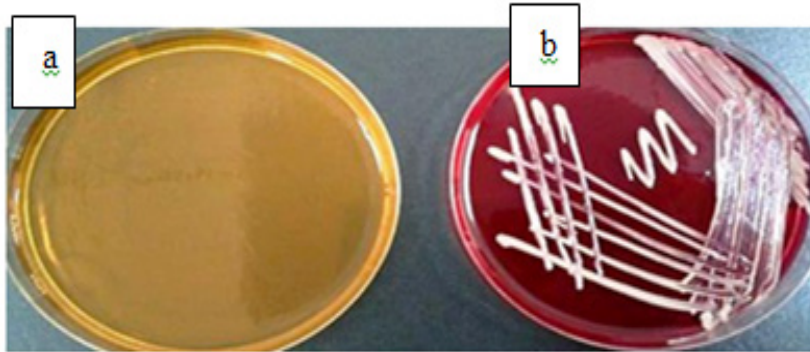


Figure 1. a- Non inoculated brain heart infusion agar. **b-** *S. aureus* produces enterotoxine.

Plasmids profile analysis

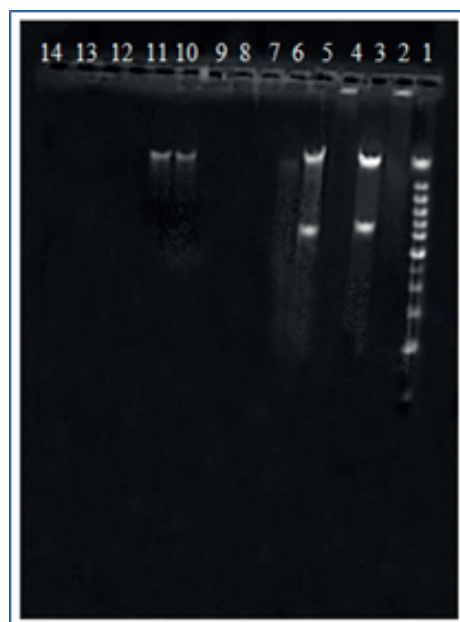
Plasmid DNA was extracted from *S. aureus* isolates by alkaline lysis¹³. Investigation was done in order to find relationship between enterotoxin and genetic determinants. The results revealed presence of (80 and 55) kb DNA plasmids in staph no.89 isolate and (80) kb DNA plasmid in staph no.81 isolate as showed in figure 2 (lane: 3 and 10). *S. aureus* no. 89 and 81 isolates were tested for transferability of their DNA plasmids to (DH10B *E. coli*) which were obtained from Science and Health Research Center (SHRC) of Koya University. Transformation process was done successfully as showed in figure 2 (lane: 5 and 11).

The transformed cells were re-streaked onto fresh selective brain heart infusion agar and the results showed positive results after 24 hrs incubation at 37oC by changing the color of the media from yellow to red, for isolates no. (11, 81, 88 and 89). The results showed that enterotoxin genes of four of the isolates which are located on the plasmids among nine Enterotoxigenic *S. aureus*, as showed in figure (2-B). And, the identities of this four isolates were re-confirmed on the basis of their morphology characteristics and biochemical activities as mentioned before. While, DH10B *E.coli* doesn't have enterotoxin gene as showed in figure 3 (lane 9) and remain yellow as showed in figure 2A.



Figure 2. A- DH10B *E. coli* on enterotoxin selective media. B- Staph no. (11, 81, 88, and 89) after transformation process to DH10B *E. coli* on selective media.

The plasmids were then photographed and UV transillumination was used. 1Kb DNA marker was used to determine or measure the size of the plasmid as standard, as demonstrated in figure 3 (lane 1).



Electrophoreses run at 75V for 1:30hrs.

Lane1: DNA marker 1Kb.

Lane3: *S. aureus* isolate No.89.

Lane5: *S. aureus* isolate No.89 after transformation process to DH10B *E.coli*.

Lane7: *S. aureus* isolate No.89 after curing by methanol extract of clove.

Lane8: *S. aureus* isolate No.89 after curing by methanol extract of cinnamon.

Lane9: DH10B *E.coli*.

Lane10: *S. aureus* isolate No.81.

Lane11: *S. aureus* isolate No.81 after transformation process to DH10B *E.coli*.

Lane12: *S. aureus* isolate No.81 after curing by methanol extract of clove.

Lane13: *S. aureus* isolate No.81 after curing by methanol extract of cinnamon.

Figure 3. Plasmid profile of *S. aureus* isolates no. (89 and 81) before transformation process, after transformation to DH10B *E.coli*, DH10B *E.coli* and after curing by (*E. caryophyllata* and *C. zeylanicum*).

Curing of plasmid DNA which has carried enterotoxin gene in *S. aureus* isolates by some medicinal plant extracts (*E. caryophyllata* and *C. zeylanicum*)

The methanol extract of *E. caryophyllata* and *C. zeylanicum* at sub minimum inhibition concentration (sub MIC) (100 and 5000) $\mu\text{g/ml}$ respectively. Results showed the genes responsible for relasing enterotox-

ins and located on plasmids were affected by plant extracts; as showed in figure 2 (lane 3): staph no.89 have two plasmids with molecular weight of the large one is about 80 Kb and the other is 55 Kb. Lane 7 and 8: is staph no.89 after treating with *E. caryophyllata* and *C. zeylanicum*, methanol extracts at concentration (100 and 5000) $\mu\text{g/ml}$ respectively affected the genes responsible for producing enterotoxins and eliminated both plasmids. Lane 9: is DH10B *E. coli*.

Lane 10: is staph no. 81 which has one plasmid with molecular weight about 80kb. Lane (12 and 13): Is staph no. 81 after treating with (*Eugenia caryophyllata* and *Cinnamomum zeylanicum*) at concentration (100 and 5000) µg/ml respectively eliminated the plasmid with the genes responsible of producing enterotoxins.

DISCUSSION

In total, 100 BacT/ALERT positive blood culture bottles 25 of them were identified as *S. aureus*, this results agree with the study of (24) that showed *S. aureus* bacteremia was found in nine (12%) of 77 patients at a median time of 4 days after CVC removal that obtained from blood culture. And the study of (25) demonstrated from 150 BacT/ALERT blood cultures in which a direct Gram stain showed Gram positive cocci resembling staphylococci were examined. Cultures of the broths revealed 66 positive for *S. aureus*, 81 positive for coagulase negative staphylococci, and three positive for species of micrococcus.

Then, the present study demonstrated twenty five isolates of *S. aureus* that isolated from positive blood culture bottles nine of them were observed as enterotoxin producer, this agree with the results of (26) shows that the production of enterotoxin A, B, C and D by 196 *S. aureus* strains isolated from blood cultures and 95 strains from nasal carriers was investigated. 30.6% of the bacteremia strains and 40% of the nasal strains produced enterotoxins. Enterotoxigenic strains of *S. aureus* produce at least 19 variants of enterotoxins. Another study recorded different percentage of toxin-positive isolates of *S. aureus*, the overall rate were 67.9% (27). This is not in accordance with the present study. It may be due to environments, geographical region and nutritional factors. The results determined from nine samples which they have enterotoxin four of the enterotoxin gene

isolates are located on the plasmids, and the study of (28) showed staphylococcal plasmids may also encode toxin genes. For example, a large 37.5-kb *S. aureus* plasmid, pRW001, contains genes encoding exfoliative toxin B, bacteriocin, and bacteriocin immunity. Another researcher (29) revealed in his study the results of molecular tests for the detection of genes encoding the toxins SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, SEIJ, SEIP and SER. Of the 481 strains of *S. aureus* tested, 255 (53%) were positive for one or more SE genes and 35 different enterotoxin gene. The genes encoding for SED, SER and SEIJ are carried on the same plasmid and among all the profiles, sed-ser-seij (15%) was the most common, followed by seg-sei (123%). These latter genes are likely to be carried together on the EGC. SE genes carried on plasmid and EGC were the most frequently present within the isolates bearing SE genes.

The present study demonstrated curing or eliminating those plasmids that have carried enterotoxigenic genes by *E. caryophyllata* and *C. zeylanicum* methanol extracts. This agree with the study of Abhinav *et al.*, (8) who illustrated that *S. aureus* produces a wide range of exotoxins that facilitate its pathogenesis in the host. Plant compounds at their sub-inhibitory concentrations were found to modulate toxin production in *S. aureus*. Essential oils from clove and cinnamon reduced *S. aureus* alpha-hemolysin, enterotoxin A and enterotoxin B production in vitro. Also, Cruickshank *et al.*, (30) reported similar findings and observed that eugenol (active component in clove oil) significantly reduced the production of alpha hemolysin, enterotoxin A and B, and toxic shock syndrome toxin in vitro. There are many studies about eliminating or affecting on plasmid with its virulence factors such as (biofilm, resistant genes, enterotoxin genes and others). The study of Adel and Srwa (31) used three medicinal plants watery and

alcohol extracts for eliminating resistant genes of *E. coli*, also the study of Muhammad *et al.*,⁽³²⁾ used three medicinal plant extracts for curing resistant genes of *Salmonella spp.*

CONCLUSIONS

This study shows that only nine isolates of enterotoxigenic *S. aureus* were isolated from 25 coagulase positive *S. aureus* in the 100 positive blood culture bottles. The genes of four of the isolates which were responsible for producing enterotoxin are located on plasmids, these plasmids were transferred to DH10B *E. coli* successfully by transformation process. Then, SMIC (sub minimum inhibition concentration) of methanol extracts of medicinal plants (*Eugenia caryophyllata* and *Cinnamomum zeylanicum*) were acting as curing agent on the enterotoxin gene.

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