The Effect of *Thymus vulgaris* Extracts on Enterotoxins (A-D) Production by *Staphylococcus aureus* Isolated from Urinary Tract Infection

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Abstract

One hundred and eighty five urine samples were collected eight isolates (4.3%) were obtained and diagnosed as *Staphylococcus aureus*. The identification of all *S. aureus* isolates was confirmed by conventional biochemical tests. Among 8 isolates, 5 (62.5%) *S. aureus* isolates were found to be enterotoxigenic . Approximately most of isolates produced at least two types of staphylococcal enterotoxins (SEs). These enterotoxins may be important in the physiopathology of urinary tract infection . The production of staphylococcal enterotoxins (A–D) in the presence or absence of *Thymus vulgaris* the crude extracts (aqueous and alcoholic) were was estimated in microtiter plates using a reversed passive latex agglutination (SET-RPLA) kit . The extracts reduced enterotoxin production at subminimal inhibitory concentrations compared with the control. At 400 µg/ml , total enterotoxin inhibition was observed for enterotoxin C production, whereas production of enterotoxins A, B, and D were totally eliminated at 800 µg/ml . The results show that the aqueous and alcoholic extracts from the leaves of *T. vulgaris* significantly decreased, in a dose-dependent manner, the production of SEA, SEB,SEC and SED by *S. aureus* .

Keywords: *Staphylococcus aureus* , Staphylococcal enterotoxins, *Thymus vulgaris* , urinary tract infection.

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تأثير المستخلصات الخام لعشبة الـ *Thymus vulgaris* على السموم المعوية (A-D) المنتجة من جرثومة الـ *Staphylococcus aureus* المعزولة من اصابات الجهاز البولي

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الخلاصة

جمعت 185 عينة ادرار وتم الحصول على ثمان عزلات جرثومية (4.3 %) شخصت على انها تعود للنوع *S. aureus*. وقد تم تشخيص كل العزلات الجرثومية بالاعتماد على الاختبارات البيوكيميائية. خمسة من هذه الثمان عزلات الجرثومية (62.5%) لها القابلية على انتاج السموم. وقد وجد ان غالبية العزلات الجرثومية لها القابلية على انتاج على الاقل نوعين من السموم. هذه السموم ربما يكون لها دور في امراضية الجرثومة في اصابات الجهاز البولي. تم تقييم انتاج السموم المعوية بوجود مستخلصات خام (مائية وكحولية) لعشبة *T. vulgaris* باستخدام تقنية الـ reversed passive latex agglutination . وقد لوحظ ان هذه المستخلصات اختزلت انتاج السموم بالمقارنة مع مجموعة السيطرة. وعند التركيز 400 ميكروغرام / مليلتر لوحظ تثبيط كلي لانتاج السموم من نوع C، بينما لوحظ تثبيط كلي للسموم من نوع A و B و D عند التركيز 800 ميكروغرام / مليلتر. وعليه تظهر النتائج بان المستخلصلت المائية والكحولية من اوراق عشبة *T. vulgaris* لها القابلية على اختزال انتاج السموم من نوع A و B و C و D المنتجة من جرثومة *S. aureus*.

الكلمات المفتاحية: المكورات العنقودية، السموم المعوية، اصابات الجهاز البولي

Introduction

Staphylococcus aureus is a human pathogen encapsulated bacterium with anti-phagocytic activity, which can invade and survive within a wide variety of mammalian cells (1). These bacteria are a leading cause of both community- and hospital-acquired infections associated with significant morbidity and mortality. This pathogen causes a wide spectrum of clinical illnesses, including skin and soft tissue lesions, diarrhea, urinary tract infections and lethal infections such as osteomyelitis, endocarditis, pneumonia and septicemia (2,3,4). *S. aureus* should be considered a pathogen in urine and that clinicians should be aware that the isolation of *S. aureus* from urine samples places patients at higher risk for eventual *S. aureus* bacteremia(5) postulated that (1) *S. aureus* urinary tract infection was a true clinical entity, and (2) staphylococcal bacteremia could result from urinary tract colonization with *S. aureus*. Bacteriuria with *S. aureus* is postulated to occur through a limited number of

echanisms–primarily ascending spread after instrumentation (e.g., urologic procedures or urethral catheterization) orhematogenous seeding of the genitourinary tract (6). For the majority of diseases caused by *S. aureus*, pathogenesis depends on the ability of the strain to survive, multiply under a variety of conditions and produce various extracellular compounds. Haemolysins, nuclease, coagulase, lipase, toxic shock syndrome toxin 1 (TSST-1), protein A and staphylococcal enterotoxins (SEs) are among the extracellular toxins and enzymes produced by *S. aureus* (2,7,8). SEs have the immunomodulatory properties of superantigens, stimulating release of T-cell-derived cytokines and T-cell activation . The SEs form a family of major serological types of heat-stable enterotoxins (9) and, thus, hinder the development of protective immunity, while romoting the persistence of bacteria in the host (1). Todate, a number of SEs have been identified, including SEA-E, SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN and SEO (2,7,8,10). The genus *Thymus* contains about 350 species of aromatic perennial herbaceous plants and sub shrubs 40 cm tall in the family Lamiaceae (11,12).Thyme (*Thymus vulgaris*) is a pleasant smelling perennial shrub, which grows in several regions in the world (Mediterranean region including Iraq, Asia, Southern Europe and North Africa) (13,14). The antioxidant, immunological, inflammatory activities of *T. vulgaris* have been reported earlier. Furthermore, the antimicrobial activities of essential oils of *T. vulgaris* have also been investigated and revealed to be active against a number of microorganisms using various assays (15,16) The chemical composition of *T. vulgaris* essential oils which is rich in thymol (29.74%) and p-cymen (30.26%). Thymol and carvacrol are the main phenolic compounds responsible for most of the therapeutic properties (13) . The compounds which comprise the essential oil of *T. vulgaris* have been identified as phenoylic compounds such as tymol (44.4-58.1%), carvacrol (2.4-4.2%) and γ-terpipene (6.9-18.9%). These compounds have strong antibacterial effects and are also found in the extracted water soluble fraction of thyme (17). The search on the crude solvent extracts of *Thymus* sp. is limited. However, there were no data on enterotoxins production by *S. aureus* exposed to extracts prepared from *T. Vulgaris*. The present study was aimed to determine the prevalence rate and nterotoxigenecity, of *S. aureus* isolates recovered from urinary tract infections in Babylon, Iraq. Addition to investigate the influence of subinhibitory concentrations of aqueous and alcoholic extracts prepared from the

leaves of *T. Vulgaris* on the production of SEs (A – D) from these bacteria as this has not been investigated previously.

Materials and Methods

Bacterial isolates

This study was done in Technical Collage \ Al-Musayib, during the period between April to November, 2014.Eight isolates of *S. aureus* were isolated from urine samples out of 185 patients suffering from urinary tract infection in Babylon city hospitals, Iraq. Urine samples were streaked onto the surfaces of mannitol salt agar, MacConkey agar, and blood agar and incubated at 37°C for 24 hours,depending on cultural, morphological and biochemical analysis according to (3,5,18). These include gram staining, catalase reaction, production of acid from mannitol salt agar, anaerobic fermentation of mannitol, production of acetoin, and tube coagulase test moreover API Staph system was performed. The bacterial isolates were maintained on agar slants. Each culture was activated by transferring a loopful from brain heart infusion (BHI) slants into nutrient broth (10 ml) followed by incubation at 37°C for 24 and subcultures were freshly prepared before use. The organisms were stored in trypticase soy broth (TSB), to which 15% glycerol was added at -20°C (4).

Assay of enterotoxins production

The production of enterotoxins A to D in the presence or absence of *T. vulgaris* extracts was estimated by reversed passive latex agglutination with the SET-RPLA kit, defined as a good method for culture filtrates . In this assay, latex particles, sensitized with the antibodies of staphylococcal enterotoxins, agglutinate in the presence of the corresponding enterotoxin. All *S. aureus* isolates were incubated into TSB with shaking aerobically at 37°C for 24-48 h. The cultures were centrifuged at 900 *g* for 20 min at 4°C and filtered through a 0.45 µm low protein-binding membrane filter. Microtitre plate was arranged so that each row consisted of 8 wells, and five rows of such 8 wells were used for each isolate. Twenty five microlitre of the diluent was dispensed into each well of the 5 rows, and 25 µl of the culture was added to the first well of each of the five rows. Doubling dilutions were performed along each of the five rows. The dilution was stopped at the 7th well so that the last well contained only the diluent. Latex

sensitized with anti-enterotoxin (A-D) supplied with the kit was added into each well appropriately and the content was mixed. Positive and negative controls were included for each sample and the plates were incubated for 20 –24 h at room temperature. The agglutination reactions were classified as positive, according to the manufacturer's instructions, when showing complete agglutination (+ + +) or incomplete agglutination with a small pellet visible in the centre of the agglutinated latex (+ + , +). Reactions were considered negative in the absence of agglutination (–). The enterotoxin titre was expressed as the reciprocal of the last dilution which gave an agglutination reaction (7).

Plant material and preparation of crude extracts from *T. vulgaris*

The extraction of *T. vulgaris* leaves was prepared according to the method of (19). The leaves of *T. vulgaris* were collected from local market with a highly degree of quality assurance and the species were identifying in the herbarium of Babylon University. The plant material was thoroughly washed with clean water to remove soil and other dirt.The air-dried plant materials were ground into fine powder in grinder and extracted with 95% ethanol and distilled water by maceration. A 100g sample of ground plant was soaked in 500 ml solvents (water for aqueous extraction and ethanol for alcoholic extraction) at 25ºC for 7 days . The extracts were filtered through a Buchner funnel evaporator at 40ºC to facilitate their further freeze-drying process. The concentrated extracts were finally freeze-dried at -50ºC for 24 hr and stored at -20ºC. The extracts were redissolved in their solvents before each individual experiment .

Preparation of extracts stock solutions

One hundred mg/mL of each extract was dissolved in its solvent, sterilized through a 0.22 µm Millipore filter, and kept as stock solution. final prepared two fold concentrations of 100, 200, 400 and 800 µg /ml. stock solutions at various concentrations were prepared in dimethyl sulphoxide (DMSO) (19).

Preparation of microbial suspension

Stock culture for each isolates of *S. aureus* inoculum were kept on nutrient agar (NA) slant under refrigeration (7 °C±1 °C). Inocula used in the assays were obtained from overnight cultures grown on NA slant at 37 °C. A loopful of the culture was diluted in sterile

saline solution (0.85 g/100 mL) to have a final concentration of approximately 108 colony forming unity per mL (cfu/mL) adjusted according to the turbidity of 0.5 McFarland standard tube. Final concentration of the inoculum in the medium used for the minimum inhibitory concentration(MIC) assays was approximately 1.5 x 108 cfu/mL (20).

Determination of the minimum inhibitory concentration(MIC) of the crude extracts

Sterile 96-well plates were used to determine minimum inhibitory concentration (MIC) through micro broth dilution. In this method, 75 µl of a 0.5 McFarland standard suspension of the bacterium (equivalent to 1.5 x 108 cfu/ml) was added to a microwell containing 75 µl of 100-800 µg/ml crude extracts in Muller-Hinton Broth. Control organism suspension and the culture medium were dispensed into one row and crude extracts with different concentrations were added to one column. The microplates were incubated for 24 hours at 37ºC (21). The supernatant was taken and submitted to enterotoxins detection test according to the procedures described by the manufacturer. Results were expressed as positive (+) and (−) negative enterotoxin production. The lowest concentration of crude extracts required to completely inhibit the enterotoxins production of the tested microorganism was designated as the MIC. Tubes without crude extracts were tested as positive control assay (20).

Statistical analysis

All experiments (MIC determination, growth experiments and enterotoxin detection) were repeated three times (triplicate) . The experimental results were expressed as the mean ± standard deviations (SD). Statistical differences were examined using the independent Student t-test. A p value less than 0.05 was considered to be statistically significant.

Results and Discussion

Isolation and identification of bacterial isolates

Eight cultures of *S. aureus* recovered from 185 urine samples (4.3 %). The identification of all *S. aureus* isolates was confirmed by conventional biochemical tests (Table 1). The API Staph. system was performed to support the identification process. *S. aureus* is a common pathogen in the community and in hospitals. *S. aureus* causes significant mortality and morbidity but is an infrequent cause of urinary tract infection(6) . However it is a primary

urinary pathogen among long-term care patients (5). Nearly similar results were previously reported by Gad et al. (4) who isolated *S. aureus* from urinary tract catheterized patients with a percentage of 6.2%. In addition, 100 strains of *S. aureus* were obtained over a four-month period from urinary tract infections was reported by Moussa et al.(22). The results are dissimilar to the results of other study by Chihara et al. (6) which showed that *S. aureus* is rarely isolated from urine; it accounts for only 1% to 1.5% of positive urine cultures. In a multicenter, community-based study conducted in Great Britain, *S. aureus* accounted for only 0.5% of isolates (20). A similar laboratory-based study conducted in France found that *S. aureus* accounted for only 1.3% of isolates from urine specimens submitted from the community (23) . While Muder et al. (5) which showed that 1.7% of *S. aureus* isolates isolated from urine samples.

Table 1. Biochemical characterization of *S.aureus* isolates isolated from urinary tract infection samples.

Biochemical test	Reaction
Oxidase	-
Catalase	+
coagulase test	+
Urease hydrolysis	+
Acid from sugar	
Glucose	+
Lactose	+
Mannitol	+
Maltose	+
Sucrose	+
Indole production	-
Methyl red	+
Voges- Proskauer	+
Citiate utilization	-
Urease	+

Staphylococcal enterotoxins (SEs) assay

All *S. aureus* isolates obtained in this study were investigated for their ability of SEs (SEA, SEB, SEC and SED) production by using the reverse passive latex agglutination (RPLA)

method . Five out of 8 (62.5%) *S. aureus* isolates were found to be enterotoxigenic (5 isolates had the ability to produce enterotoxin**)** , three of them produced two types of SEs, one of them produced three types of SEs, and one of them produced only one type of SEs (Table 2). Two isolates (25%) were found positive on the presence of SEA (T1 and T6), two isolates (25%) produced SEB only (T3 and T4)**,** also two isolates (25%) produced SED (T4 and T6**),** while four isolates (50%) produced SEC. The isolate (T1) produced both SEA and SEC, while the isolate (T3) produced both SEB and SEC, also the isolate (T4) produced SEB and SED, while the isolate (T6) produced SEA, SEC and SED together, and the isolate (T8) produced SEC only. As SET-RPLA, which is the most common laboratory method for detection of SEs from bacterial strains, is designed to detect only classical SEs (SEA-SED), underestimation of potentially SE producing isolates may be expected (22,24). In this study, 62.5% of isolated *S. aureus* strains produced classical enterotoxins. Other studies (25,26) which showed that 45% of *S. aureus* strains isolated from skin infection were capable of producing staphylococcal enterotoxins. Solano et al, (27) showed that 19.04% of the *S.aureus* strains isolated from vomit samples were enterotoxinogenic. Al- Jumaily et al. (28) showed that 50.8% of *S. aureus* isolated from mastitis in cows had the ability to produce enterotoxin. In this study most of the isolated strains produced SEC. These results are in agreement with (29) which showed that 23.8% *S. aureus* strains isolated from various clinical samples (blood and urine) produced SEC, while dissimilar with the same study (29) reported that none of the strains produced SEA, SEB and SED. Variation in the results reported in other studies may be a result of different sampling techniques employed, seasonal effects, and/or laboratory methodologies employed in different studies.

Table 2. SEs production from *S. aureus* isolates isolated from urine samples by RPLA method.

Isolates	Staphylococcal enterotoxins (SEs)			
	SEA	SEB	SEC	SED
T1	+	_	+	_
T2	-	-	-	-
T3	-	+	+	
T4	-	+	-	+
T5	-	-	-	-
T6	+	-	+	+
T7	-	-	-	-
T8	-	-	+	-

The variation of staphylococcal isolates in their ability to produce enterotoxins depend on the source . The host plays an important role in assisting an adaptation between the bacteria and their surrounding environment (30). For example most of the bacteria isolated from milk and mastitis in cows produce SEA (28,31,32). While bacteria isolated from dairy products produce SEC (33) and most bacteria isolated from skin and human wounds produce SEB (25, 26) .

Effects of extracts from *T. vulgaris* on Staphylococcal enterotoxins (SEA – SED)

Evaluation of toxin production (SEA-SED) by *S. aureus* in the presence of *T. vulgaris* extracts (aqueous and alcoholic extraction) revealed that the ability to produce toxin decreases as extracts concentration increases. Both the extracts of *T. vulgaris* have shown inhibit SEs production at different concentrations compared to the control . Especially, alcoholic extract showed good inhibitions against SEs production . Aqueous extract of *T. vulgaris* showed moderate inhibition against SEs production . At subinhibitory concentrations, the extract could inhibit enterotoxin production compared to the control titre value despite the slight reduction observed in the bacterial growth (Tables 3-6). Enterotoxins production were observed at MIC but at very low titre value compared with the controls. However, Table 3 revealed that 400

µg/ml of the alcoholic extract (MIC) could inhibit enterotoxin C production and at 2MIC there were no enterotoxin production from all the *S. aureus* tested in the presence of viable cells. At lower concentrations (100 µg/ ml), the bacterial growth was only slightly lowered, as compared with controls. Levels of SEC were at least fourfold lower than those recovered in the control, when 100 µg/ ml alcoholic extract were used. According to *t* test results, the extracts exhibited different effects on the enterotoxin production ($P \leq 0.05$).

Table 3. Effect of *T. vulgaris* (aqueous extraction) on Staphylococcal enterotoxins (SEB and SEC) produced by *S. aureus* T3.

Extract (µg/ml)	Viable count log(cfu/ml)	Staphylococcal enterotoxins (SEs)	
		SEB titre[1]	SEC titre[1]
0	9.87	128	128
100	8.95	64	\32
200	8.61	16	16
400	7.72	8	4
800	3.55	ND[2]	ND[2]

1 Reciprocal of last dilution that produced an agglutination.

2 Not detected.

Limit of detection of the RPLA test is 0.5 ng/ml.

Table 4. Effect of *T. vulgaris* (alcoholic extraction) on Staphylococcal enterotoxins (SEB and SEC) produced by *S. aureus* T3.

Extract (µg/ml)	Viable count log(cfu/ml)	Staphylococcal enterotoxins (SEs)	
		SEB titre[1]	SEC titre[1]
0	9.87	128	128
100	8.95	32	16
200	8.61	8	4
400	7.72	4	ND[2]
800	3.55	ND[2]	ND[2]

1 Reciprocal of last dilution that produced an agglutination.

2 Not detected.

Limit of detection of the RPLA test is 0.5 ng/ml.

The continued emergence of multiple-antibiotic-resistant *S. aureus* isolates originating from community and nosocomial sources necessitates the development of new and improved antimicrobial agents for the prevention and treatment of these life-threatening infections . To date, many studies have focused on naturally occurring compounds . Plants contain a number of organic components including alkaloids, flavones, phenols, quinones, terpenoids, and tannins, all of which are known to possess antibacterial activity (34). The results MIC of *T. vulgaris* extracts are within the range that has been reported by other researchers where medicinal plants have been shown to possess inhibitory staphylococcal enterotoxins production. Evaluation of enterotoxins (A-D) produced by *S. aureus* in the presence of *T. vulgaris* extracts revealed that the ability to produce toxin decreases as extract concentration increases.

Table 5. Effect of *T. vulgaris* (aqueous extraction) on Staphylococcal enterotoxins (SEA and SED) produced by *S. aureus* T6.

Extract (µg/ml)	Viable count log(cfu/ml)	Staphylococcal enterotoxins (SEs)	
		SEA titre[1]	SED titre[1]
0	9.33	128	128
100	8.47	64	64
200	7.84	32	16
400	7.09	16	4
800	3.96	ND[2]	ND[2]

1 Reciprocal of last dilution that produced an agglutination.

2 Not detected.

Limit of detection of the RPLA test is 0.5 ng/ml.

Table 6. Effect of *T. vulgaris* (alcoholic extraction) on Staphylococcal enterotoxins (SEA and SED) produced by *S. aureus* T6.

Extract (µg/ml)	Viable count log(cfu/ml)	Staphylococcal enterotoxins (SEs)	
		SEA titre[1]	SED titre[1]
0	9.43	128	128
100	8.86	32	32
200	8.61	16	8
400	6.03	4	2
800	3.55	ND[2]	ND[2]

1 Reciprocal of last dilution that produced an agglutination.

2 Not detected.

Limit of detection of the RPLA test is 0.5 ng/ml.

On the other hand, an alternative strategy that is now gaining interest to treat with *S. aureus* infections is the targeting of bacterial virulence factors (e.g. haemolysins, enterotoxins, adhesins) . A number of virulence factors secreted by *S. aureus* play a significant role in pathogenesis. Therefore, the clinical performance of antibiotics used for the treatment of *S. aureus* infections not only depends on the respective bacteriostatic or bactericidal effects but also on the ability to prevent the release of virulence factors by dying or stressed bacteria (2,9) . However, further studies are required into the clinical application of these herbs. Successful and standardized results may indicate usefulness of these herbs assupplement medicines in treating infections caused by *S. aureus* .

Conclusion

S. aureus is a cause of urinary tract infection among patients. the findings in the present study that the extracts of *T. vulgaris* significantly reduces the production of key pathogenicity factors by *S. aureus* ,namely the enterotoxins (A-D). These data suggest that The extracts of *T. vulgaris* may be useful for the treatment of *S.aureus* infections. It could be suggested to use in various pharmaceutical applications.

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