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RESEARCH ARTICLE

Efflux Pump Inhibitory Potentials of Thymoquinone against Staphylococcus Aureus Isolates from Mastitis Milk

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Abstract

Staphylococcus aureus(*S. aureus*) is a common bacterial cause of bovine mastitis known for its resistance to antimicrobials through various mechanisms includingdrug efflux pumping, which aids in its colonization in theudder tissue and spread in the environment. Thymoquinone (TQ) extracted from Nigella sativa(N. sativa), is recognized for its strong antibacterial properties against S.aureus.This study aimed to evaluate the effect of TQ on drug efflux pumping in S. aureus. Herein,we collected 67 milk samples from cows with chronic mastitis to isolate S. aureus, assess its resistance to antimicrobials, and to analyze the relationship between TQ's minimum inhibitory concentration (MIC) and its inhibition zone diameters (IZDs) using the disc diffusion, as well as ethidium bromide cartwheel (EtBr CW) and accumulation assays. Out of the 67 samples, 16 (23.9%) yielded S. aureus isolates, which exhibited high resistance to several antimicrobials, with the highest resistance (87.5%) against each of cefepime and azithromycin. There was no correlation between the MIC values of TQ and the IZD for discs containing 50 µg of TQ. The efflux assay demonstrated that TQ functions as an efflux pump inhibitor (EPI) similar to verapamil for some S. aureus isolates.The study concluded that the antibacterial effectiveness of TQ is largely due to its role in inhibiting drug efflux pumps, a mechanism prevalent among the tested isolates and comparable to that of verapamil. Additionally, MIC detection could not reliably predict the results from disc diffusion testing.

Keywords: Thymoquinone, Efflux pump inhibitors, Nigella sativa plants, Antibiotic resistance

Introduction

The advent of antibiotics brought a major breakthrough in medicine during the 20th century, but it wasn't long before resistance started to develop. Although new antibiotics have been created in response, bacterial resistance has continued to grow, with various mechanisms evolving to defeat these drugs. Resistance has emerged across nearly all classes of antibiotics, leading to many bacterial strains becoming resistant to multiple drugs. These drug-resistant bacteria present serious risks, leading to severe infections in both medical and veterinary environments[1].

Bacteria employ a range of tactics to resist antibiotics, such as altering or breaking down the drugs, changing the target they aim at, shielding the target from the drug, or reducing the drug's presence within the cell by restricting its entry or actively expelling it[2, 3].Efflux pumps in bacteria are involved in removing harmful internal metabolites, releasing virulence factors, and managing

cellular stress. As a result, antibiotics can unintentionally become targets for these transporters[4, 5].These membrane proteins bind to substrates, including antibiotics, and actively transport them out of the cell. This action lowers the intracellular levels of these substances, thereby diminishing the effectiveness of the treatment[2,6].*Staphylococcus aureus(S. aureus)* both plasmids and chromosomes contain more than 15 genes encoding efflux pumps. These include *NorA, NorB, NorC, MdeA,* and *SdrM* from themajor facilitator superfamily (MFS), as well as *MepA* from the multidrug and toxic compound extrusion (MATE) family. These pumps play a key role in multidrug resistance (MDR) by helping to expel macrolides, tetracyclines, and fluoroquinolones, thus aiding in the removal of toxic compounds[7]**.**

The rise in antibiotic-resistant bacteria highlights the need for new treatment approaches. One promising strategy is to use efflux pump inhibitors (EPIs) in conjunction with antibiotics to address resistance caused by efflux pumps[8].EPIs are compounds that can block efflux pumps, thereby boosting the concentration of antibiotics inside bacterial cells. Verapamil, an ion channel blocker commonly used to manage hypertension, has been shown to improve the effectiveness of ofloxacin and bedaquiline by inhibiting the activity of MATE pumps[9]**.**For the treatment of *S. aureus* osteomyelitis, diclofenac sodium, a non-steroidal anti-inflammatory medicine (NSAID) with FDA approval has been used as an adjuvant anti-virulence therapy in conjunction with antibiotics [10]. It has been discovered that the antiemetic drug dopamine has other medicinal uses. It improved the efficiency of levofloxacin and ciprofloxacin in the fight against MDR*Escherichia coli*(*E. coli*)[11].

Nigella sativa (N. sativa), an annual spice belonging to the *Ranunculaceae* family, linn is most extensively researched for its medicinal properties[12].Owing to these benefits, *N. sativa* seed and oil are used worldwide to treat a wide range of illnesses, including hemorrhoids, icterus, asthma, diarrhea, dyspepsia, fever, apoplexy, and disorders of the cardiovascular, digestive, respiratory, kidney, and immune systems. The majority of this plant's medicinal qualities are a result of thymoquinone (TQ), a significant active ingredient in the essential oil[13].

Nigella sativa essential oil (EO) and its compounds (thymoquinone, carvacrol, and p-cymene) have a broad antimicrobial spectrum It was proved on this study was to investigate the antimicrobial and resistance modifying activity of *N. sativa* EO, thymoquinone, carvacrol, and p-cymene against one methicillin susceptible and one methicillin resistant *S. aureus* strain. *N. sativa* EO, thymoquinone, carvacrol, and p-cymene were assessed for antimicrobial activity and modulation of antimicrobial resistance (by broth microdilution), inhibition of antimicrobial efflux (by ethidium bromide [EtBr] accumulation assay), relative expression of *mepA* gene (by real-time reverse transcriptase quantitative polymerase chain reaction), membrane disrupting effect (by LIVE/DEAD BacLight™ Kit), and finally antibiofilm activity (by the crystal violet assay). Both strains of *S. aureus* were susceptible to *N. sativa* EO, thymoquinone, and carvacrol. *N. sativa* EO and carvacrol induced the increase of EtBr accumulated by both *S. aureus* strains. Membrane integrity of ATCC strain was disrupted by carvacrol and p-

cymene, whereas for the methicillin resistant *S. aureus* (MRSA) strain the membrane integrity was disrupted by each compound. N. sativa EO and its bioactive compounds such as carvacrol and p-cymene could be applied as resistance modifiers in MRSA strains[14].

One of the following techniques can be used to assess a drug's efflux potential: i) detecting fluorescent dye extrusion from bacterial cells directly [15] ii) usingmass spectrometry- or liquid chromatography-based tests to quantify the amount of medication that has accumulated inside bacterial cells [16], iii) usingethidium bromide (EtBr) and agar-based techniques to demonstrate the efflux pump activity[17], iv)identifying interactions between EPIs and their targets by in silicodocking, then validating the results *in vitro* using fluorEscence-based ethidium accumulation tests or MIC determination (checkerboard synergy assay)[18]andv) utilizing real-time PCR to identify the way inhibitory substances alter the activity of the efflux pump gene[19].Because medicinal plants include a wide variety of secondary metabolites with a range of pharmacological characteristics, they are extremely attractive sources of natural EPIs. Studies on these plant extracts have shown putative compounds that prevent efflux pumps in bacteria, both positive and negative[20].It has been determined that secondary metabolites isolated from *Artemisia absinthium* function as anti-biofilm agents preventing *E. coli* and *E. faecalis* from producing MFS pumps. Furthermore, it has been demonstrated that ciprofloxacin's effectiveness can be increased by extracts from the medicinal plant *Myristica fragrans*, which prevent 80% ofmethicillin resistant *S. aureus*(MRSA) growth by altering the expression of the *norA* and *mepA* genes[21]**.**The objective of the current investigation was to assess thymoquinone's EPI capability against milk-derived MDR *S. aureus* isolates.

Materials and methods

Bacterial isolation:

In Sharkia Governorate, Egypt, 67 milk samples were collectedfrom cows suffering from chronic mastitis at different locations. Each sample was cultured for 24 hours at 37°C. After that, mannitol salt agar (HIMEDIA,India) was loaded with a loopful of the resultant broth culture and incubated using the same parameters. Suspected colonies were cultivated on brain heart infusion (BHI) agar (HIMEDIA,India)and kept for a full day at 37°C in the incubator. The tube coagulase test was then used to determine if rabbit plasma had coagulated in the colonies that had formed, in accordance with established procedures [22].

Identification of bacteria

Using Blue Staph Latex Kits from Pro-Lab Diagnostics and adhering to the manufacturer's instructions, a single colony from the pure culture was examined for the presence of clumping factor and protein A. For later usage, coagulase-positive bacteria were kept in 10% glycerol at -80°C.

Antimicrobial susceptibility test (AST) of *S. aureus* isolates

Antimicrobial susceptibility testing was conducted on the identified *S. aureus*isolatesadoptingthe previously published protocol[23] using the specified antibiotic discs(Bioanalyse,Turkyia)**:**amoxicillin(AM), oxacillin(OX), amoxycillin/clavulanic acid (AMC),doxycycline(DO),ampicillin/sulbactam (SAM), cefepime(CFP), linezolid (LZD), trimethoprim/sulfamethoxazole

(SXT), chloramphenicol(C), azithromycin (AZM), ciprofloxacin(CIP),gentamicin (CN), and clindamycin[24-26].

Determination of minimum inhibition concentration (MIC) and inhibition zone diameters of thymoquinone

Sigma provided 99% pure thymoquinone (TQ) (catalog number 274666-5G, Sigma, St. Louis, MO, USA). Ten percentof dimethylsulfoxide(DMSO) was used to dissolve 250 mg of TQ, which was then kept as a stock solution at -20°C. After being diluted in 1.0 milliliter of a 1:1 ethanol/water mixture, this stock solution was employed at the necessary concentration. Thymoquinone (TQ) was tested for its anti-efflux impact on *S. aureus* by measuring its minimum inhibitory concentrations (MICs) against ten strains of the bacteria using the Clinicaland Laboratory Standards Institute-recommended broth microdilution method [24].To make the *S. aureus* suspensions, the broth was diluted to $5x10^5$CFU/mL. A sterile 96-well microtiter plate was filled with equal volumes (100 µL) of the bacterial culture and serial dilutions of TQ in broth, with final TQ concentrations of 0 (control), 1, 2, 4, 8, and 16 µg/mL. After adhering tape to the plates, they were incubated at 37˚C for 24 hours. As the lowest concentration of TQ that prevents observable bacterial growth, the MIC was determined. Every isolate underwent independent testing in triplicate. Using Mueller-Hinton Agar (MHA) supplemented with 1% glucose, the antibacterial susceptibility of *S. aureus* strains to TQ was evaluated in accordance with a conventional procedure [22]. Discs were also fixed on the agar and loaded with 20 µl (50 µg TQ) dissolved in 10% DMSO and alcohol. Control discs containing 10% DMSO were included. The culture plates were then incubated at 37 °C for 24 hours, and inhibition zone diameters (IZDs) were measured in millimeters.

Ethidium bromide agar cartwheel method (EtBr CW)

S. aureus isolateswith a high resistance profile were grown onto BHI agar with different concentrations of EtBr (0.5 - 2.5 mg/L) in order to evaluate the bacteria' phenotypic efflux pump activity using the EtBr CW method**.**Freshisolates(10^7 CFU/mL)were distributed radially on each plate and injected in a cartwheel pattern onto plates containing varying doses of EtBr. Onto EtBr-agar plates, cultures were injected, extending outward toward the margins from the center. For the EtBr efflux activity, both positive and negative control strains were included on each plate. Following an overnight incubation period at 37°C, the plates were examined using a UV transilluminator. Fluorescence is a sign of efflux pump activity in strains of bacteria that are resistant to drugs (MDR). Several strains' EtBr efflux activity of TQ (0.5 MIC) was assessed.

Ethidium bromide accumulation assay

The ethidiumbromide accumulation assay was used to compare the anti-efflux activity of TQ to that of verapamil (VP), as previously described[23]**.** Briefly, 10^6CFU of *S. aureus* was suspended in 100 µLof BHI containing 1% glucose and a 0.5 MIC-TQ in microtiter plates and incubated for 15 min at 37˚C with EtBr (10 µg/mL). The same procedure was undertaken using VP (200 mg/L) as a positive control. Intracellular accumulation of EtBr was assessed using a Synergy HT reader (Biotech) with excitation at 490 nm and emission at 579 nm after 15, 30, and 45 minutes. The average readings of three measurements were documented. An increase in fluorescence intensity indicated EtBr

binding to the DNA of *S. aureus*, while a decrease in fluorescence over time indicated EtBr being extruded.

Results and Discussion

The most common Gram-positive bacterium, *S. aureus*, is linked to both clinical and subclinical mastitis. It is also a major cause of morbidity and mortality worldwide, causing a variety of illnesses in humans, from minor skin infections to life-threatening sepsis and pneumonia [27-28]. The emergence of MDR strains of *S.aureus* makes treating these infections more challenging; hence, there is an urgent need to discover effective medicines against this pathogen. The creation of novel antibiotics or the restoration of the efficacy of presently available antibiotics can impede the emergence of bacterial resistance. Sixteen (23.9%) *Staphylococci* isolates out of67 milk samples collectedfrom cows suffering from chronic mastitis were isolated and recovered on mannitol salt agar. These isolates were then identified as *S. aureus* using tube coagulase and clumping factor detector. Previously, the recovery rate of *S. aureus*(22–60%)isolatedfrom cow`s milkreflects variations in farm management techniques, geography, and detection methodologies[29-33]..

Out of the 67 isolates that were sent for the subsequent investigations, 16 isolates were chosen for the current study, which showedhigh resistance to several tested antimicrobial drugs (Table 1). Cefepime and azithromycin had the highest resistance percentage (87.5%), while trimethoprim/sulfamethoxazole and clindamycin showed a little higher proportion. It was shown that doxycycline, linezolid, and chloramphenicol exhibited minimal levels of resistance (37.5 %). Due to the extensive antimicrobial resistance of *S aureus* to β-lactams, difficult-to-eradicate *S. aureus* strains have emerged [34-35].

The result reported above revealed that *N. sativa* oil has antibacterial activity against *S. aureus*. The agreement between the commonly used simple disc diffusion test for evaluation of *N. sativa* EO and TQ was investigated in comparison with the reference broth microdilution (BMD) method outlined by the Clinical and Laboratory Standards Institute (CLSI) (Table 1)IZDs of disc diffusion and MICs of *N. sativa* EO and TQ by broth microdilution against *S. aureus* strains obtained from different sources are shown in Table 1**.**There was no observed correlation between the MIC readings of EO and TQ and the corresponding IZDs for the same strains when using 20 mg dry matter/disc and 50µg/disc, respectively.

The MICs results of disc diffusion could not be distributed in categorized in relation to IZDs. Six resistant strains to TQ with high MICs ranged from 160-320 µg/ml showed IZDs ranged from 20-40 mm by disc diffusion. This suggests poor growth poor growth of TQ resistant strains onto MHA medium (Table 1). Similarly, four strains resistant to *N. sativa*EO with high MICs ranged from 66-269 mg dry matter/ml showed IZDs of the same strains ranged from 15-20 mm by disc diffusion, indicating poor growth of *N. sativa* EO resistant strains onto MHA medium.

Table (1) Susceptibility of *S. aureus* strains to different antimicrobials and thymoquinone

Strain Code	Antibiotics													TQ
	SAM	AMC	OX	FEP	CN	LNZ	C	SXT	DO	CIP	DA	AZM		IZD/ MIC (µg /ml)
1			R	R				R	R	R	R	R		26/160
2	R	R	R	R				R	R	R	R	R		19/160
3	R	R	R	R										10/320
4	R	R	R	R							R	R		40/5
5			R					R		R	R	R		26/40
6	R	R		R	R			R		R	R	R		29/40
7	R			R						R		R		20 /20
8			R	R		R	R	R	R	R	R	R		40/80
9	R	R		R	R	R	R	R			R			40/320
10	R	R		R		R		R		R		R		40/160
11	R	R	R	R	R			R	R	R	R	R		15/40
12	R	R	R	R	R	R	R	R		R	R	R		30/160
13	R	R		R	R	R	R	R	R		R	R		15/160
14		R					R	R			R	R		40/80
15	R	R	R	R	R	R	R	R	R	R	R	R		15/160
16	R		R	R	R			R			R	R		40/20
Resistance %**	**75**	**68,7**	**62.5**	**87.5**	**43.7**	**37.5**	**37.5**	**81.3**	**37.5**	**62.5**	**81.3**	**87.5**		

Resistant(R),Ampicillin/sulbactam(SAM), amoxycillin/clavulanic acid(AMC), cefepime(FEP), gentamicin(CN) , linezolid(LZD), Chloramphenicol (C), trimethoprim/ sulphamethoxazol(SXT), Doxycycline (DO) Ciprofloxacin(CIP), Clindamycin(DA), Azithromycin (AZM), Oxacillin(OX) Clindamycin(DA) and Thymoquinone (TQ). *Multiple Antibiotic Resistance (MAR) index is calculated by dividing the number of antibiotics an organism is resistant to by the total number of antibiotics it has been exposed to.**Resistance % is calculated by dividing the number of resistant isolates by the number of tested isolates or each antibiotic and multiplied by 100. Empty cella indicate sensitive strains to the tested antimicrobial.

Following the evaluation of the MIC and IZDs of TQ for 18 *S. aureus*isolates, no association was found between the MIC values and corresponding inhibition zone diameters (IZDs) of TQ when 50 µg per disc was used as shown in linear regression analysis . More specifically, the disc diffusion MIC results could not match the IZDs. Regarding TQ, four resistant bacteria exhibiting IZDs between 20 and 40 mm,those have high MICs (160–320 µg/mL) (Table 1). A representative sample for MIC determination is shown in Figure 1. Disc diffusion was a commonly employed method to evaluate the antibacterial efficacy of TQ and essential oils (EO). There has never been any research done on the connection between MICs and agar diffusion.

These results meant that the calculated IZDs could not be used to forecast unknown MIC values. Ciprofloxacindisc diffusion was also impractical in another investigation since the agar screen plates did not grow vancomycin-intermediate resistant *S.aureus* (VISA) isolates and did not correctly designate them as susceptible[36].

When TQ is applied to bacterial cell envelopes of strains that have TQ-MIC values below the pharmacokinetic blood maximum concentration (C-max, 3.48 µg/mL), TQ is directly bactericidal. However, similar antibacterial effects are not observed in isolates whose TQ-MIC values are greater than twice this concentration. Only one isolate out of 16 was deemed sensitive (5 µg/mL) in the circulation based on TQ pharmacokinetic parameters in this study since MICs ranged from 5 to 320 µg/mL. a similar vein, a previous research [37] presentedthat while the majority of *S. aureus* strains were sensitive to TQ-EPI, just one isolate (4 µg/mL) in the bloodstream was determined to be sensitive based on TQ C-max, with sensitivity reliant on the anti-efflux activity of 0.5 MIC-TQ. TQ therefore shows minimal accumulation in target tissues, poor pharmacokinetics, and low bioavailability. Encapsulated nano-delivery methods have the potential to improve pharmacokinetics by raising TQ blood C-max levels. As presented previously [38], the creation of nanostructured carriers for TQ has proven successful.

Rats were given a single oral dose of TQ (20 mg/kg) loaded into nanostructured lipid carriers (TQ-NLC), which produced a C-max of 3348 ng/ml, considerably higher plasma concentrations, and faster absorption (1 hour) than an unloaded TQ suspension (1160 ng/mL). [39]

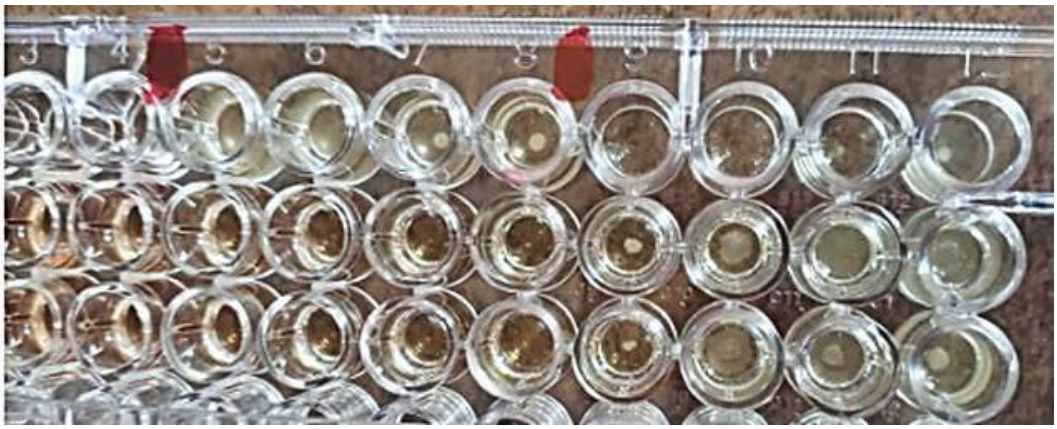

Figure 1 Representative sample for MIC of TQ.First row (A1-A8 positive control culture, A9-A12 negative control culture. Second and third row (MIC of TQ of a *S.aureus* strain showing clear wells until

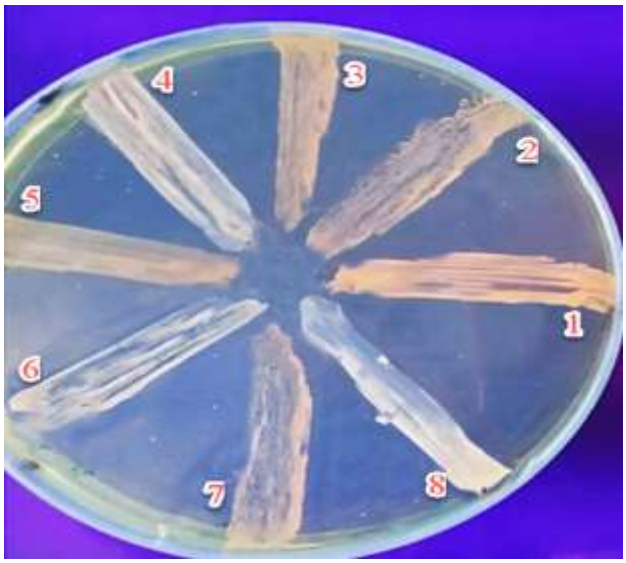

Figure 2 **:** Representative plate of cartwheel method showing 7 tested isolates and one standard *S.aureus* strain at EtBr (0.5mg/L)under a UV transilluminator. Positive fluorescence (indicating negative efflux) was observed in isolates 2, 3, 5, 7and the standard isolate (1), while negative fluorescence (indicating positive efflux) was seen in isolates 4, 6, and 8.

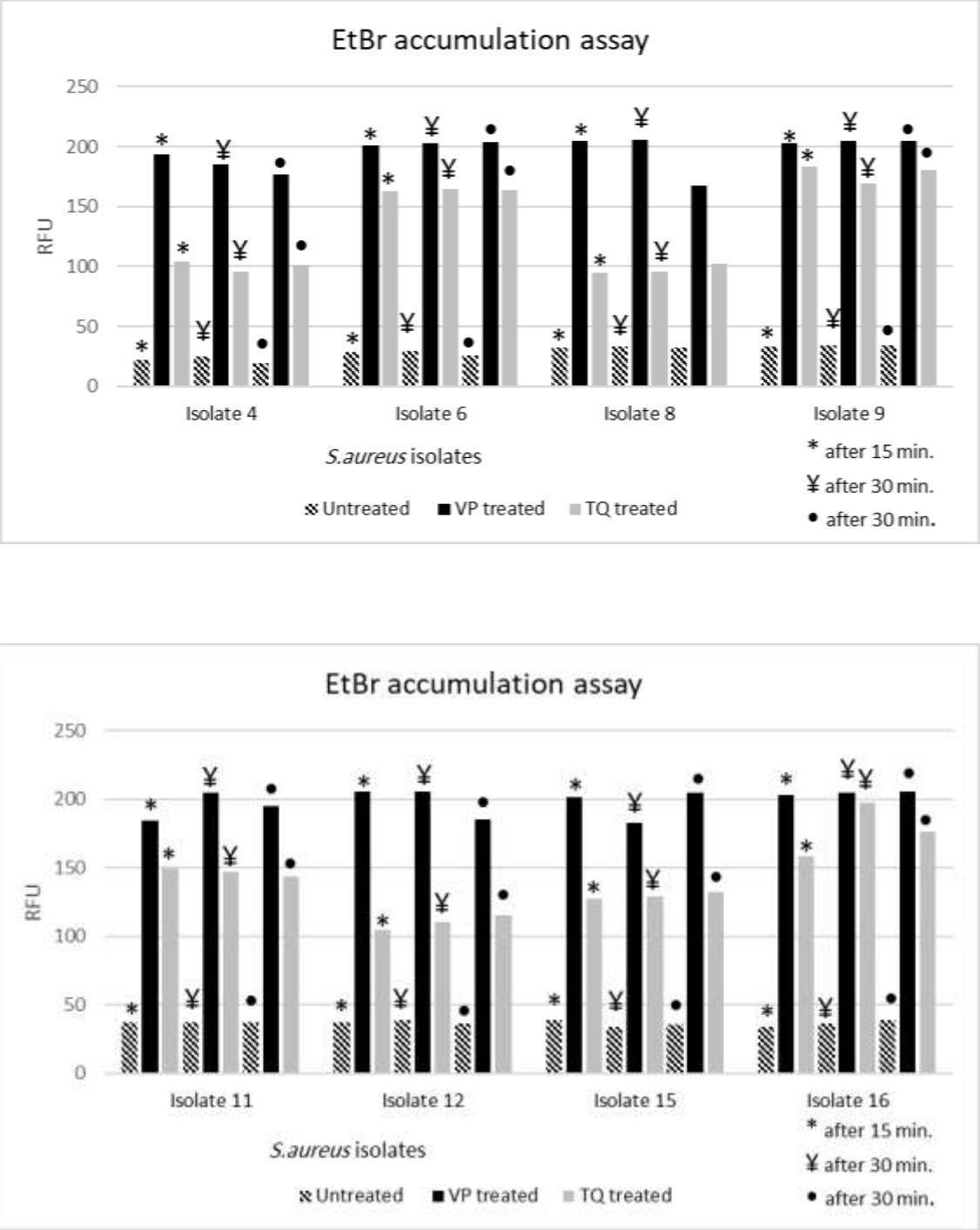

Figure 3 **:**Fluorometric EtBr accumulation assay of 8 isolates after treatment with thymoquinone and verapamil Results are expressed as relative fluorescence unit on the longitudinal axis for each isolate after exposure to thymoquinone and verapamil for 15 min.(*) , 30 min.(¥) and 45 min. (•) in addition to RFU of the untreated same isolates.

Using the Cart-Wheel method, the efflux of EtBr in the absence of TQ was assessed on the sixteen isolates of *S. aureus* that were chosen. A typical sampling of the eight tested isolates' varied efflux capabilities at EtBr 0.5 mg/L is shown in Figure 2. An EtBr accumulation assay with fluorometric detection was used to assess TQ's anti-efflux efficacy on the eight isolates that were chosen. Units of relative fluorescence (RF) are used to express the results. A decrease in fluorescence is indicative of increased efflux pump activity in the isolate, which is indicated by a lower RF. On the other hand, larger RF values relative to the control (verapamil) suggest that the tested drug has more efflux inhibitory efficacy. According to the results of the efflux experiment, TQ has EPI activities that are lower in the remaining isolates and comparable to verapamil in some isolates (6, 9, 11, and 16) are shown in Figure 3. Even within the same species, bacterial populations frequently display genetic variability in terms of their EPI capacities. Variations in the genes encoding efflux pumps or the regulatory proteins regulating their expression can result from mutations and horizontal gene transfer.Furthermore, certain environmental factors, such the presence of heavy metals, antibiotics, or other stressors, can favor bacterial isolates with improved efflux capacities. In some isolates, this selection pressure may be what propels the emergence of highly effective efflux systems[40-41].

Linear regression analysis of IZDs produced by 20mg/disc and MICs of N. sativa EO were conducted (Figure 4A). Similarly, IZDs produced by 50µg of TQ/disc were plotted against TQ-MICs(Figure 4 B). Scalar readings of IZDs showed no linear correlation with the MICs readings of CLSI broth microdilution (BMD). Linear regression analysis revealed that unknown MIC values could not be predicted from the estimated zone diameters. Linear regression analysis of IZDs and corresponding MICs of both N. sativa EO and TQ was invalid.[42].

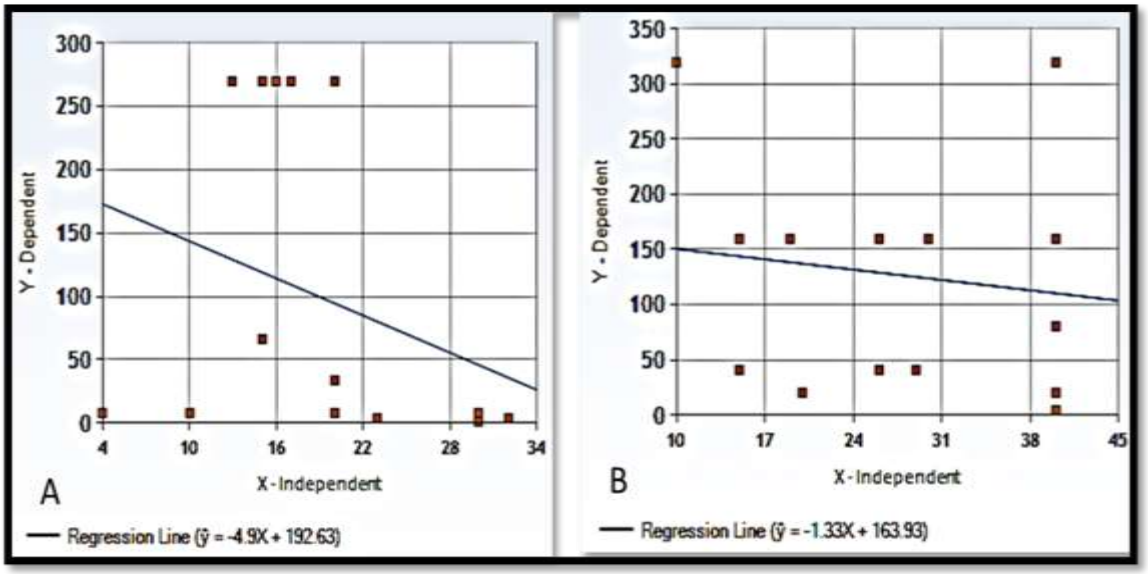

Figure 4 (A, B) Regression lines of N. sativa essential oil (A) and thymoquinone (B) of 18 S. aureus strains plotted against inhibition zone diameter (X- axis) obtained with 20 mg N. sativa dry matter/disc and 50 µg thymokinon/disc. Invalid regression lines showed lack of correlation between inhibition zone diameters and MICs (Y axis) of N. sativa essential oil or thymoquinone.[42].

Conclusion

Thymoquinone demonstrated a high MIC in its antibacterial activity. The measurement of TQ-MIC against *S. aureus* was effective in evaluating TQ's antibacterial properties. The results of the disc diffusiontechnique made it impossible to anticipate the MIC values of TQ. The potential of TQ as an efflux pump inhibitor (EPI) differed amongst *S. aureus* isolates.

Ethical approval

NA

Data availability

All data generated or analyzed during this study are included in this published article and available on request.

Conflict of interest

The authors declare no competing interests.

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الملخص العربى

القدرة المثبطة لمضخة التدفق للثيموكينون ضد عزلات المكورات العنقودية الذهبية

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المكورات العنقودية الذهبية هي سبب بكتيري شائع لإلتهاب الضرع البقريحيث إنه يقاوم مضادات الميكروبات بآليات مختلفة، وخاصة ضخ تدفق الدواء الذي يساعد على استعمار أنسجة الضرع ونشرها لاحقًا في المناطق المحيطة. يُعرف الثيموكينون (TQ) المشتق من مستخلص حبة البركة، بأنه منتج نباتي قوي مضاد للبكتيريا ضد المكورات العنقودية الذهبية.

أجريت هذه الدراسة للتحقق من ضخ تدفق الدواء الناتج عن للثيموكينون ضد المكورات العنقودية الذهبية. تم تجميع 67 عينة لبن لعزل المكورات العنقودية الذهبية ومعرفه ملامح المقاومة لمضادات الميكروباتوارتباط قراءات TQ-MIC وانتشار القرص، وعجلة بروميد الإيثيديوم(EtBr CW)، وتحقيقات تراكم بروميد الإيثيديوم. من بين 67 عينة لبن تم جمعها من الأبقار المصابة بالتهاب الضرع المزمن، تم عزل 16 (23.9٪) عزلة من المكورات العنقودية الذهبيةوأظهرت جميعها مستويات مقاومة عالية للعديد من العوامل المضادة للميكروبات التي تم اختبارها، مع ملاحظة أعلى نسبة مقاومة (87.5٪)ضدإزيثروميسين. لم يلاحظ أي ارتباط بين قيم MIC للثيموكينون وأقطار منطقة التثبيط المقابلة له (IZDs)، عند استخدام 50 ميكروجرام لكل الأقراص التي تم فحصها. كشف اختبار التدفق أن TQ له أنشطة مثبط مضخة التدفق (EPI) مماثلة لتلك الخاصة بالفيراباميل ضد بعض عزلات المكورات العنقودية الذهبية. وتوصلنا إلى أن النشاط المضاد للبكتيريا لـ TQ يعتمد بشكل أساسي على تدفق المضخة الدوائية، والذي كان سائداً بين العزلات المدروسة وكان مشابهاً لفعالية الفيراباميل. لا يمكن التنبؤ بالحد الأدنى من اكتشاف التركيز المثبط كطريقة مرجعية من نتائج انتشار القرص المقدرة.