Glyceryl trinitrate blocks staphyloxanthin and biofilm formation in *Staphylococcus aureus*

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Abstract

Background: *Staphylococcus aureus* is an important nosocomial bacterium that is responsible for a number of infections that may be fatal. The treatment of such infections is limited by emergence of antibiotic resistance. Targeting virulence of *Staphylococcus aureus* may provide an alternative option to antibiotic that may bypass the emergence of resistant strains due to lack of stress on viability.

Objectives: Investigation of the ability of glyceryl trinitrate (GTN) to inhibit staphyloxanthin, biofilm and tolerance to oxidative stress.

Methods: The disk sensitivity method was used to detect the methicillin resistance of *Staphylococcus aureus*. The effect of sub-inhibitory concentration of GTN on biofilm formation, staphyloxanthin production and tolerance to oxidative stress was evaluated. Molecular docking study was used to investigate the ability of GTN to bind to dehydrosqualene synthase enzyme.

Results: GTN showed a significant inhibition of biofilm, staphyloxanthin and tolerance to oxidative stress. In the molecular docking study, it was found that GTN could bind to dehydrosqualene synthase enzyme by hydrogen bonding,electrostatic interaction and pi-cation interaction.

Conclusion: The present study revealed the ability of GTN to serve as a potential anti-virulence candidate for attenuation of *S. aureus* pathogenicity.

Keywords: Glyceryl trinitrate, *Staphylococcus aureus*, staphyloxanthin, biofilm.

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Introduction

Unlike the large number of antibiotics discovered or synthesized in the twentieth century,the number of antibiotics developed in the current century is much lower[1]. Moreover, antibiotics prescribed for treatment of infections over many years led to the emergence of antibiotic resistant strains of *Staphylococcus aureus* due to the stress exerted on bacterial growth[2,3]. One approach of combating antibiotic resistance is based on disarming the bacterial virulence factors that enable the bacteria to cause disease. This strategy avoids the bacterial cell viability-stress induced emergence of resistance and, as a result, can be helpful in preventing or treating infections[3,4].

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S. aureus is a human pathogen that is responsible for several nosocomial and community-acquired infections such as cutaneous and soft-tissue infections in addition to dangerous systemic infection[5-7]. Methicillin-resistant *S. aureus* (MRSA) is a great public-health problem[8-10]. MRSA are resistant to the majority of available antibiotics and the treatment options are very few[11].

In order to develop multidrug resistance, it employs an arsenal of virulence factors that help it evade the host immune response. Staphyloxanthin; the golden yellow carotenoid pigment is one of such virulence factors. It acts as a reducing agent to neutralize the reactive oxygen species (ROS) produced by neutrophils and macrophages[12, 13]. Without staphyloxanthin, *S. aureus* is defective in infectivity and is liable to be attacked by neutrophils with the subsequent inability to cause infection in a mouse model[1,5,14].

Staphylococcus aureus has the ability to form biofilms either on human tissues or implants, resulting in chronic infections that are difficult to treat due to extreme resistance to antibiotics[15]. Inhibition of staphyloxanthin was previously investigated[16].

Glyceryl trinitrate (GTN) is a drug used in treatment of hypertension[17]. GTN was able to inhibit biofilms formed by *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *candida albicans*[18]. GTN is approved by FDA for topical treatment of anal fissures at concentrations up to 0.4%[19].

The objective of this study was to identify the potential inhibitory activity of glyceryl trinitrate against staphyloxanthin and biofilm formation.

Materials and methods

Media and chemicals

The media used in this study were Mueller Hinton broth, Mueller Hinton agar, Tryptone soya broth and Tryptone soya agar. These media in addition to methicillin antibiotic disk were purchased from Oxoid (Hampshire, UK). Glyceryl trinitrate was obtained from POHL-Boskamp, Gmbh &Co., Hohenlockstedt, Germany (Stock solution of 1 mg/ml). Other chemicals were of pharmaceutical grade.

Bacterial strains

Staphylococcus aureus ATCC 6538 strain was kindly provided by the Department of Microbiology, Faculty of Pharmacy, Mansoura University. A clinical isolate of *Staphylococcus aureus* (SA1) was isolated from a patient with a surgical site infection at Zagazig University Surgery Department and identified by The MALDI-TOFF apparatus at the Clinical Pathology Department, Faculty of Medicine, Zagazig University.

Antimicrobial susceptibility testing

Antibiotic susceptibility of SA1 was tested against methicillin using the Kirby-Bauer standard disk diffusion method according to CLSI guidelines[20].

Müeller-Hinton broth (5 ml) was inoculated with three to five well-isolated colonies from an overnight agar plate culture and the broth culture was incubated at 37°C with shaking for 4 to 6 hours until a turbidity of a 0.5 McFarland standard was achieved or exceeded. Sterile broth was used to adjust the turbidity to achieve turbidity matching that of a 0.5 McFarland standard. A sterile cotton swab was moistened with the bacterial suspension and pressed on the inside wall of the tube to remove excess inoculum. The swab was streaked both over the surface of the Mueller Hinton agar plate and around the agar rim. The antibiotic disks were put on the inoculated plate and gently pressed into the agar and the plates were incubated at 37°C for 18 hours. The inhibition zones diameters were measured, and interpreted as resistant, intermediate or susceptible according to CLSI[21].

Determination of Minimum Inhibitory Concentration (MIC)

The agar dilution method was used in determination of the minimum inhibitory concentration of GTN according to the Clinical Laboratory and Standards Institute Guidelines (CLSI)[20]. The tested strain was incubated overnight in tryptone soya broth (TSB) and the suspension was diluted with Mueller-Hinton broth in order to prepare a suspension with a turbidity approximating that of 0.5 McFarland Standard. The suspension was further diluted with sterile saline (1:10). By using a micropipette, a standardized inoculum (approximately 10^4 CFU per spot) was spotted on the surface of Mueller-Hinton agar plates containing different GTN concentrations and control plate without GTN. The MIC of GTN was the lowest concentration that inhibits growth on the plate after incubation at 37 °C for 20 hours.

Assay of biofilm formation and inhibition

In order to test the biofilm formation capacity of *S. aureus*, the modified method of Stepanovic et al.[22] was used.

From overnight cultures of the tested strain, bacterial suspension with a density of 1×10^6 CFU/ ml was prepared by dilution with TSB. The bacterial suspension was delivered in aliquots (100µl) to a sterile microtiter plate wells that was incubated for 48 hours at 37°C. The non-adherent cells were discarded by removal of the suspensions from the microtiter plate wells and the wells were then washed 3 times using sterile phosphate buffered saline. The biofilm cells were fixed by 99% methanol for 20 minutes and the wells were stained with crystal violet (1%) for another 20 minutes followed by washing with distilled water for removing excess dye.

After leaving the plate to air dry, the dye was dissolved by 33% glacial acetic acid and the contents of the microtiter plate were transported to another plate for measuring the optical densities at 590 nm using spectrofluorimeter (Biotek, USA).

The cut-off optical density (ODc) was determined as three times standard deviations above the mean OD of the negative control. Following the criteria of Stepanovic et al.[22], *S. aureus* strain was non-biofilm producer (OD less than ODc), weak biofilm producer (OD between ODc and 2x ODc), moderate biofilm producer (OD between 2x ODc and 4x ODc), or strong biofilm producer (OD more than 4x ODc).

In order to investigate the biofilm inhibiting activity of GTN, the previous procedure was repeated, but in the absence and presence of 0.125 mg/ml of GTN and biofilm inhibition was calculated by the following formula

% of biofilm inhibition = (OD without GTN-OD with GTN)/ OD without GTN

Microscopic visualization of biofilm inhibition

S. aureus was grown in TSB and incubated overnight at 37°C and the optical density was adjusted to reach 1 at 600 nm. Sterile glass cover slips were placed inside in 50 ml falcon tubes containing fresh media and inoculated with the bacterial suspension in the presence and absence of 0.125 mg/ml of GTN. The biofilms were allowed to form for 48 hours at 37°C and the cover slips were washed and stained with crystal violet (1%) and examined under the light microscope using the high power (400X magnification)[22].

Staphyloxanthin assay

The ability of GTN to reduce the production of the golden yellow staphyloxanthin pigment was investigated1. For qualitative assay, overnight culture of the tested strains in MHB was prepared. The bacterial suspensions were diluted 1:100 in fresh MHB containing GTN and control tubes without GTN were prepared in the same way. The tubes were incubated for 24 hours at 37 °C with shaking at 250 rpm. The pellets were collected by centrifugation at 16,600×g for 10 min and the pellets were photographed and the golden yellow pigment was compared in treated and untreated samples.

For quantitative assay, the pellets recovered were resuspended in methanol (0.2 ml) by vortexing, and the resultant mixture was heated at 55°C for 30 minutes for pigment extraction. The extracted pigment was separated by centrifugation at 16,600 xg for 10 minutes and the extraction process was repeated thrice. The absorbance of the extracted pigment solutions was measured at 465nm using spectrofluorimeter (Biotek, USA).

Sensitivity to oxidative stress

To investigate the interfering capacity of GTN with resistance to oxidative stress, the modified disk assay method of Hassett et al.[23] was used. *S. aureus* was cultured in TSB and incubated overnight. Aliquots of 0.1 ml were spread onto the surface of Tryptone soya agar plates with and without 0.125 mg/ml of GTN. Sterile paper disks (6 mm) were placed on the surface of Tryptone soya agar plates and 10 µl of hydrogen peroxide (1.5%) was added to them. The inhibition zones were measured after incubating the plates for 24 hours at 37°C.

Docking study

The crystal structure of the C30 carotenoid dehydrosqualene synthase from *Staphylococcus aureus* was retrieved from Protein Data Bank (PDB ID: 2ZCQ)[24]. Docking study was carried out on glyceryl trinitrate (GTN) into the receptor active site using Molegro Virtual Docker (MVD Version 6.0). GTN was drawn into Marvin Sketch V5.11.5[25]. The most energetically favored conformer was exported as mol^2 file format for docking. The optimal geometry of the ligand was determined during the docking process. The search area was set to be 13 Å from the center of the active site. MolDock optimizer algorithm was chosen to perform docking process with 10 runs per ligand, 150 population size, 4000 max iteration and 8 poses for each ligand. MolDock score[26] was used as scoring function. Finally, the top returned poses were chosen for analysis.

Statistical analysis

One Way ANOVA followed by Newman-Keuls Multiple Comparison Test, Graph Pad Prism[5] was used to investigate the significance of the inhibitory activities of GTN against staphyloxanthin, oxidative stress and biofilm formation. P values <0.05 were considered statistically significant.

Results

Methicillin resistance of SA1and antibacterial activity of GTN against *S. aureus*

The clinical isolate of *S. aureus* SA1 was found to be resistant to methicillin. GTN inhibited the growth of *S. aureus* at 0.5 mg/ml. The inhibitory activity of GTN against biofilm formation, staphyloxanthin production and resistance to oxidative stress was evaluated at ¼ MIC (0.125 mg/ml).

GTN inhibits biofilm formation

The standard strain *S. aureus* ATCC 6538 is well-known as a strong biofilm forming strain. The clinical isolate was also strong biofilm producer as its OD is 0.806>0.256 (4X ODc). GTN exerted a significant biofilm inhibition in both strains. Biofilm of *S. aureus* ATCC 6538 was inhibited by 69.16%, while that of the clinical isolate was reduced by 52.64% (Fig. 1). This biofilm inhibiting activity was further confirmed by microscopical examination of biofilms formed on glass cover slips in the presence and absence of GTN and examined with the light microscope. Much fewer scattered cells were found in the sample treated with GTN.

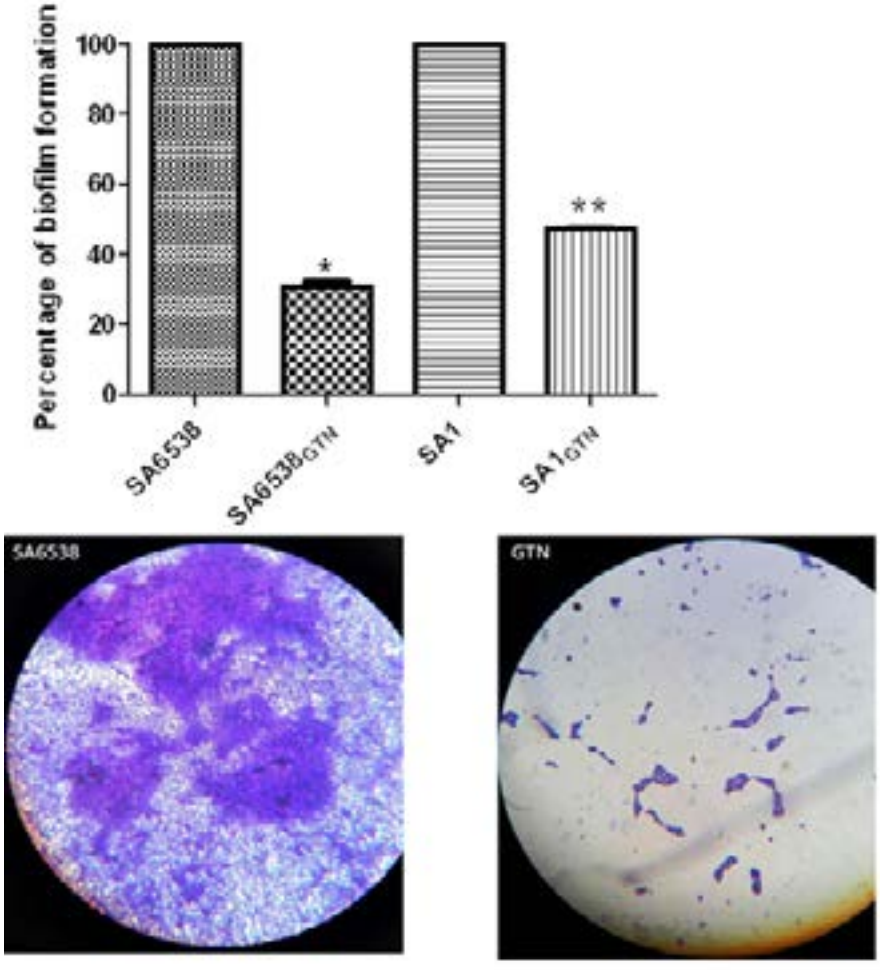

Figure 1. Inhibition of biofilm formation of *S. aueus* by ¼ MIC of GTN. *, significant P < 0.05. Microscopic images of biofilm formed by *S. aureus* ATCC 6538 under the light microscope (X400) in the treated culture (right) and untreated (left).

GTN inhibits staphyloxanthin production

The golden yellow color of staphyloxanthin enables the visual inspection of its production. The cell pellets separated from the GTN treated samples showed a remarkable reduction in staphyloxanthin production (Fig. 2). This was confirmed by quantitative assay in which a significant inhibitory activity of staphyloxanthin was found in the GTN treated samples as compared to the control samples. The staphyloxanthinproduction was reduced by 63.37% in *S. aureus* ATCC 6538 and by 70.98% in the clinical isolate SA1.

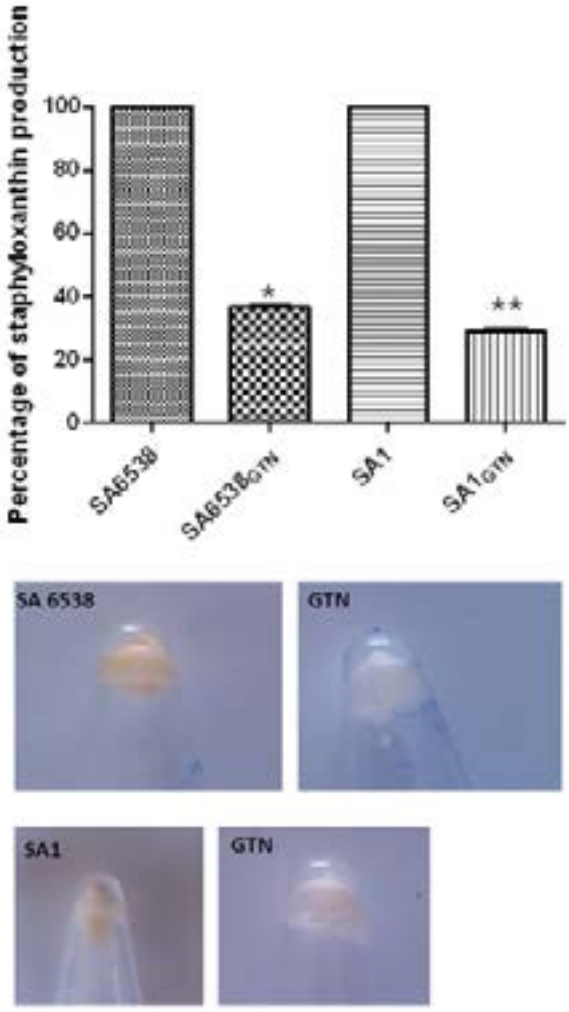

Figure 2. Inhibition of Staphyloxanthin production by ¼ MIC of GTN. *, significant P < 0.05.

GTN decreases the tolerance against oxidative stress

The inhibitory activity of GTN against the tolerance to oxidative stress was investigated by testing the augmentation of hydrogen peroxide on the growth of *S. aureus* by GTN. GTN showed a significant decrease in the tolerance to oxidative stress by 36.94% in *S. aureus* ATCC 6538 and 42.6% in the clinical isolate SA1 (Fig. 3).

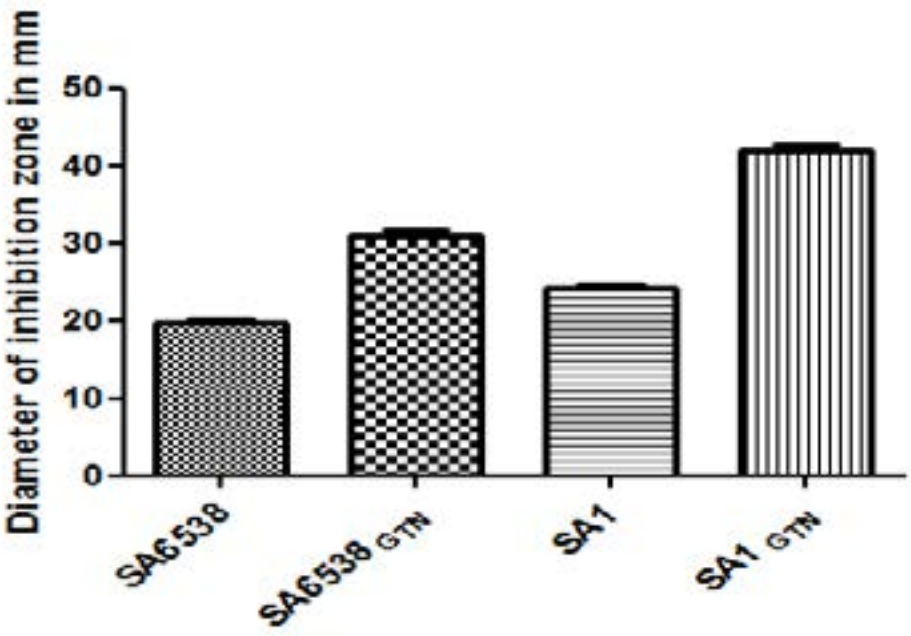

Figure 3. Reduction of tolerance to oxidative stress by ¼ MIC of GTN. *, significant P < 0.05.

Docking study

The binding mode of GTN was studied using molecular docking study. GTN has nine hydrogen bonds with Arg45, Tyr129, Gln165, Asn168, Val 133 and Tyr248 electrostatic interaction with Arg45 and Asp48 and pi-cation interaction of the nitrogen atom with Tyr183 resulting in docking score of -99.658 (Fig. 4). GTN can effectively interact with the dehydrosqualene synthase active site.

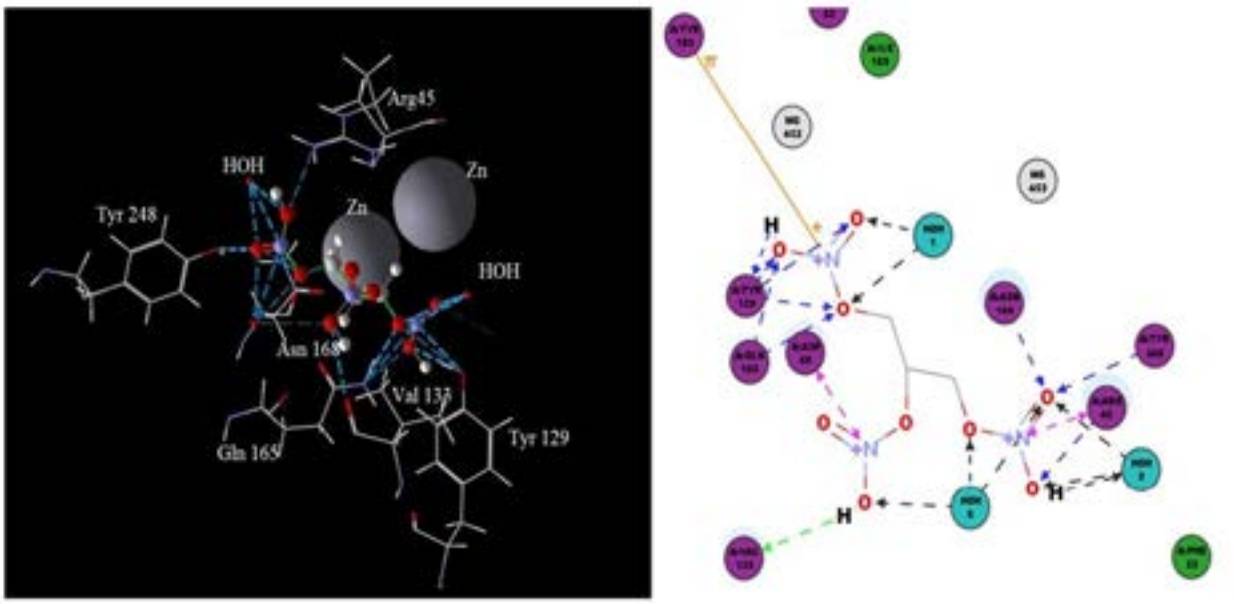

Figure 4. The Molecular docking of GTN into the active site of dehydrosqualene synthase 3D (Left) and 2D schematic view of the binding (Right).

Discussion

The rate of methicillin resistance among *S. aureus* reached a peak in such a way that the mortality rate in the U.S. due to invasive MRSA exceeds that due to AIDS[27,28]. This problem needs urgent action for development of other therapeutic approaches[29]. One of these approaches is the search for anti-virulence drugs. In our study, GTN could inhibit the growth of *S. aureus* 6538 and the clinical isolate SA1 at 0.5 mg/ml. The anti-virulence potential of GTN was investigated at 0.125 mg/ml. The capability of *S. aureus* to produce virulence factors is linked to its pathogenicity. These factors include biofilm formation and the antioxidant pigment staphyloxanthin[30].

Staphyloxanthin protects *S. aureus* from phagocytosis. It was reported that the strains of *S. aureus* that are deficient in staphyloxanthin production are sensitive to killing by reactive oxygen species produced by neutrophils and are defective in their ability to form skin abscesses[1].

GTN exerted biofilm inhibiting activity that ranged between 52.64% and 69.16%. The anti-biofilm activity of GTN against *S. aureus* was previously reported as a catheter lock solution in combination with ethanol and citrate[31]. The anti-biofilm activity of GTN was further explored by microscopic examination of biofilms formed on glass cover slips treated and untreated with GTN. The treated sample showed very few scattered cells in contrast to the dense compact cells in the control sample.

In this study, the biological activity of GTN on the production of staphyloxanthin pigment was inspected by extraction of the pigment from GTN treated and untreated cells. The pigment in GTN treated cells was reduced to the range of 63.37% to 70.98%. Furthermore, the in vitro susceptibility to oxidants was tested by investigation of the augmentation of the sensitivity to hydrogen peroxide. The susceptibility to hydrogen peroxide was increased between 36.94% and 42.6%. This may be due to blockade of staphyloxanthin pigment. This was in accordance with the finding of Liu et al.[32]

Dehydrosqualene synthase is an enzyme that is involved in the first step of staphyloxanthin biosynthesis[13]. As a result, the interaction of GTN with dehydrosqualene synthase enzyme was investigated. Docking study was made to identify the ability of GTN to bind to the active site of dehydrosqualene synthase. GTN was found to attach to the enzyme by a combination of hydrogen bonds, electrostatic interaction and pi-cation interaction. In other terms, GTN may be a competitive inhibitor of this enzyme leading to inhibition of staphyloxanthin synthesis.

Interestingly, the anti-virulence activity of GTN was exerted at 0.125 mg/ml, a much lower concentration of GTN that is approved by FDA for topical use (up to 0.4% or 4 mg/ml).

Repurposing GTN as an anti-virulence agent is advantageous. It is an FDA approved whose pharmacological and toxicological properties are well-known. Moreover, using FDA approved drug saves the high costs and shortens the long development times to produce new antimicrobials or virulence inhibitors[33]. Our previous studies showed that GTN is a potent inhibitor of quorum sensing and virulence in *Pseudomonas aeruginosa* and *Serratia marcescens*[34,35]. The rationale of repurposing drugs for use as anti-virulence agents against *Stapylococcus aureus* was previously reported. Thus, diflunisal; the non-steroidal anti-inflammatory drug was reported as a potent inhibitor of virulence in USA300 strain of methicillin-resistant *S. aureus*[36]. However, further clinical trials are necessary to test the clinical efficacy of GTN in laboratory animals and human volunteers.

Conclusion

GTN is a potential anti-virulence agent that can be useful in the treatment of *S. aureus* topical infections.

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Conflict of interest

The authors declare that they have no conflict of interest.

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