Hesperidin inhibits biofilm formation, virulence and staphyloxanthin synthesis in methicillin resistant \textit{Staphylococcus aureus} by targeting SarA and CrtM: an in vitro and in silico approach

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Abstract

Methicillin resistant \textit{Staphylococcus aureus} is considered multidrug resistant bacterium due to developing biofilm formation associated with antimicrobial resistance mechanisms. Therefore, inhibition of biofilm formation is an alternative therapeutic action to control MRSA infections. The present study revealed the non-antibacterial biofilm inhibitory potential of hesperidin against ATCC strain and clinical isolates of \textit{S. aureus}. Hesperidin is a flavanone glycoside commonly found in citrus fruit. Hesperidin has been shown to exhibits numerous pharmacological activities. The present study aimed to evaluate the antibiofilm and antivirulence potential of hesperidin against MRSA. Results showed that hesperidin treatment significantly impedes lipase, hemolysin, autolysin, autoaggregation and staphyloxanthin production. Reductions of staphyloxanthin production possibly increase the MRSA susceptibility rate to H$_2$O$_2$ oxidative stress condition. In gene expression study revealed that hesperidin treatment downregulated the biofilm-associated gene ($sarA$), polysaccharide intracellular adhesion gene ($icaA$ and $icaD$), autolysin ($altA$), fibronectin-binding protein ($fnbA$ and $fnbB$) and staphyloxanthin production ($crtM$). Molecular docking analysis predicted the ability of hesperidin to interact with SarA and CrtM proteins involved in biofilm formation and staphyloxanthin production in MRSA.

Keywords Hesperidin · \textit{Staphylococcus aureus} · SarA · qPCR · Antibiofilm · Molecular docking

Introduction

Methicillin resistant \textit{Staphylococcus aureus} (MRSA) is a Gram-positive bacterium that causes hospital acquired infections such as bacteremia, wound infections, urinary tract infections, skin infections, pneumonia, heart valve infections, burn wound infections and bone infections (Troeman et al. 2019). Initially, it was recognized as a commensal skin bacterium, but later on, it was considered a lethal pathogen due to the biofilm formation and antibiotic resistance mechanism (Lee et al. 2018). World health organization (WHO) reported that MRSA is a high priority multidrug resistant (MDR) bacterium (WHO 2015, 2017). Several virulence factors have support that antibiotic resistance mechanism in MRSA such as quorum sensing, biofilm formation, staphyloxanthin biosynthesis, efflux pump and alteration of gene expression (Jenul and Horswill 2019). Biofilm formation is a key virulence factor for the insistent survival on various surfaces, oxidative/environmental stress and the antibiotic resistance of MRSA. Generally, bacterial biofilms are easy to attach biotic/abiotic surfaces due to extracellular polymeric substances (EPSs). EPSs contain polysaccharides, proteins, extracellular DNA and lipids. And they act as a primary barrier to restrict antibiotics penetration and host immune response (Hoiby et al. 2010). Hence, bacterial biofilms are more resistant than planktonic cells (Stewarts and Costerton 2001). Furthermore, several antibiotics treatment is a fail to control MRSA infections due to the development of biofilms which leads to increasing mortality and morbidity risk in clinical settings. In MRSA, two component systems (TCS) (agr) and nucleic acid binding proteins (SarA) have been hypothesized to control virulence factor synthesis in particular, biofilm formation (Liu et al. 2016; Tan et al. 2018). Staphyloxanthin is a yellow green carotenoid
pigment responsible for the control of bacterial growth in an oxidative stress situation (Clauditz et al. 2006). Polysaccharide intracellular adhesion (PIA) is encoded by an ica locus responsible for maintaining the bacterial cell adhesion factor (Otto 2013). These virulence factors are highly interconnected with biofilm formation. Hence, targeting biofilm formation by MRSA is an alternative approach to control/prevent biofilm-associated infections (Roy et al. 2018). Promising natural antibiofilm agents have the ability to hinder biofilm formation/preformed biofilms via inhibiting virulence factor expression in bacteria (Mishra et al. 2020). Moreover, the biofilm agents will not affect the bacterial metabolic growth, which helps to reduce the chances of resistance development against antibiofilm agents (Rabin et al. 2015). Bioactive components play an important role in drug development in pharmaceutical industries; most of the bioactive components are derived from natural sources like plants and animals (Atanasov et al. 2021). Our previous studies proved that bioactive components antibiofilm potential of clinically important pathogens, including limonene, 5-hydroxymethylfurfural, quebrachitol, eucalyptol, and 1, 8-cineole against Streptococcus pyogenes, Streptococcus mutans, Staphylococcus epidermidis, Acinetobacter baumannii, Staphylococcus aureus and Pseudomonas aeruginosa (Subramenium et al. 2015; Vijayakumar and Ramanathan 2018, 2020; Karuppiah and Thirunanasambandham 2020; Vijayakumar et al. 2020; Vijayakumar and Thirunanasambandham 2021; Karuppiah et al. 2021). Hence, the identification of antibiofilm agents from bioactive components is anticipated to assist in the drug development against MRSA.

Hesperidin is a plant based bioactive components classified as a flavonoid, commonly found in citrus fruits. It has been used for its broad biological properties, such as its antimicrobial, anti-inflammation, antioxidant and anti-cancer activities (Corciova et al. 2015; Samie et al. 2018). With this backdrop, the present study aimed to determine the antibiofilm potential of hesperidin against MRSA and mainly focused on virulence gene expression underlying the antibiofilm potential of hesperidin.

Materials and methods

Bacterial strain and growth conditions

Methicillin resistant S. aureus (ATCC 33,591) was purchased from ATCC, Himedia, India and clinical isolate strains MRSAvk9 (GenBank accession number MK757651) and MRSAvk10 (GenBank accession number MK757652) were used in the present study. The strains were streaked on tryptone soya agar (TSA) plates and incubated at 37 °C for 24 h. A single isolated colony was picked and cultured in 3 ml of tryptone soya broth supplemented with 1% of sucrose (TSBS) for enhancing the biofilm formation, incubated at 37 °C for 24 h.

Phytocompound

Hesperidin was purchased from Sigma-Aldrich (India) and prepared as 10 mg/ml stock solution in methanol and stored at 4 °C.

Determination of biofilm inhibitory concentration (BIC)

Biofilm inhibitory concentration (MBIC) of hesperidin against MRSA was assessed by crystal violet (CV) quantification assay which was performed as described earlier (Karupppiah and Thirunanasambandham 2020). Briefly, 1% of MRSA culture (1.6 × 107 CFU /ml) was added to 1 ml of TSBS along with hesperidin at increasing concentrations ranging from 25 to 150 µg/ml. The wells containing 2 ml of TSBS, 1% of overnight MRSA culture with 10 µl of methanol was regarded as vehicle control to evaluate the effect of solvent on MRSA. The wells contain 2 ml of TSBS with 1% MRSA culture and 2 ml of TSBS alone were used as control and blank. The plates were incubated at 37 °C for 24 h. After incubation, the planktonic cells were removed and washed twice with sterile distilled water and allow to air dry. The biofilm attached cells were stained with CV (4% w/v) solution for 10 min and washed twice with sterile distilled water and allow to air dry. Then, the stained biofilms were eluted by 1 ml of 20% glacial acetic acid. The biofilm biomass was measured using a UV-visible spectrophotometer at 570 nm.

Microscopic analysis

To validate the hesperidin antibiofilm potential against MRSA was assessed by microscopic analysis. Briefly, 1% of overnight MRSA (ATCC-33,591), clinical isolates MRSAvk9 and MRSAvk10 were inoculated into 1 ml of TSBS medium in the 24 well plates, inserting (1 × 1) cm glass slides with presence and absence of hesperidin at 100 µg/ml concentrations and allowed to grow for 24 h at 37 °C. After incubation, the slides were washed with sterile distilled water to remove debris of medium. For light microscopy analysis, slides were stained with 0.4% crystal violet (CV) solution and observed at 400× under the light microscope. In confocal laser scanning microscope (CLSM), slides were stained with 0.1% of acridine orange solution and observed at 200× in CLSM (Karupppiah and Thirunana-sambandham 2020).
Growth curve analysis

To evaluate the effect of hesperidin on the growth of MRSA (ATCC-33,591), clinical isolates MRSAvk9 and MRSAvk10, 100 ml of TSBS broth was inoculated with 1% inoculum of MRSA in the absence and presence of hesperidin (at its BIC). Initial OD (0 h) was measured at 600 nm and the cultures were kept at 37 °C. OD values were taken at 1 h interval for 24 h and CFU/ml was calculated at 3 h interval for 24 h and the growth curve was plotted as OD against time interval along with CFU/ml (Vijayakumar et al. 2020).

Cell viability assay

Further, the cell viability of MRSAs were quantified using the XTT (2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2 H-tetrazolium-5-carboxanilide) reduction assay. Briefly, the XTT sodium salt and menadione were dissolved in sterile PBS (1 mg/ml) and acetone (1mM) respectively. For each experiment, XTT–menadione solution was prepared freshly at the ratio of 12.5:1 respectively. After incubation, both biofilm and planktonic cells were collectively removed from the hesperidin treated and untreated wells. The cells were washed twice with sterile PBS and resuspended in the 175 µl PBS with 25 µl of XTT-menadione solution. This mixture was incubated in dark at 37 °C for 8 h. The viability of tested strains was measured spectrophotometrically by the reduction of XTT-menadione into the orange colored formation at 490 nm (Vijayakumar et al. 2020).

Auto-aggregation assay

MRSA was grown in test tubes containing 10 ml of TSBS with presence and absence of hesperidin at (BIC) for 24 h at 37 °C. After incubation, the cell pellets were harvested by centrifugation at 8000 rpm for 10 min and washed thrice with PBS. Over, the pellet was resuspended with 10 ml of PBS and kept undisturbed for 24 h. After incubation, 100 µl of cell suspensions were collected from the upper portion of test tubes, the cell density was measured at 600 nm for every 3 h interval up to 24 h (Sorroche et al. 2012).

Autolysin assay

The effect of hesperidin on autolysis of MRSA was evaluated by separating pellets from treated and untreated hesperidin samples. The collected cell pellets were washed thrice with ice-cold PBS and resuspended in autolytic buffer (0.05% Triton x-100 and 0.05 M Tris-HCL in PBS) and incubated at 30 °C. The aliquots were measured at 600 nm for every 30 min interval up to 3 h (Cue et al. 2015).

Lipase assay

Effect of hesperidin on lipase production in MRSA was assessed by previous described method (Sethupathy et al. 2017). Briefly, 100 µl of cell free culture supernatant (CFCS) from control and treated MRSA cultures were mixed with 900 µl of lipase substrate buffer containing 1:9 volume of 0.3% p-nitro phenyl palmitate in isopropanol and 50 mM Na2PO4 buffer (pH 7.0) containing 0.2% sodium deoxycholate and 0.1% gumini arabicum and incubated at room temperature in dark for 1 h. Following incubation, lipase activity was terminated by adding equal volume of 1 M Na2CO3 to the reaction mixture and centrifuged at 12,000×g for 10 min. The absorbance of the supernatant was measured at 410 nm and results are expressed as a percentage of lipase inhibition.

Hemolysin quantification assay

The efficacy of hesperidin on hemolysin was measured by microdilution method as described earlier (Hollands et al. 2008). Briefly, freshly collected sheep blood was diluted with TSBS medium to a final concentration of 2% (v/v) and divided into three aliquots. The first aliquot, 10% (100 µl) of standard cell suspension of MRSA was added. To the second aliquot, 10% of standard cell suspension and hesperidin (at its BIC) were added. The hesperidin alone at its BIC was added to the last aliquot and this was considered as blank. All the tubes were incubated at 37 °C for 1 h and subsequently incubated at 4 °C for 1 h. After incubation, the tubes were centrifuged at 5000 rpm for 5 min and the absorbance of supernatants was measured at 405 nm.

Staphyloxanthin quantification assay

To evaluate the effect of hesperidin on carotenoid pigment production in MRSA was assessed by previous described method (Leejae et al. 2013). Briefly, MRSA was grown in 100 ml of TSBS along with hesperidin (at its BIC) at 37 °C for 24 h at 160 rpm. After incubation, the control and treated samples were centrifuged at 8000 rpm for 10 min, collected the pellets and washed twice with PBS. The pellets were taken for methanolic extraction by resuspending the pellets into methanol and kept in a shaker for 24 h. After incubation, the samples were centrifuged at 10,000 rpm for 10 min and the supernatant was measured at 465 nm.

H2O2 sensitivity assay

To determine the sensitivity of MRSA cells on H2O2 in the presence and absence of hesperidin the method was followed
described earlier (Valliammai et al. 2019). Briefly, control and hesperidin treated cell suspensions were centrifuged at 8000×g for 10 min and pellets were resuspended in PBS along with 1 mM H₂O₂ and incubated at 37 °C for 1 h. After incubation, the cells were serially diluted and spread on the TSA plates and incubated at 37 °C for 24 h. After incubation, the viable cells were counted manually.

**RNA extraction, cDNA conversion and real-time PCR**

To evaluate the effect of hesperidin on selected genes responsible for biofilm formation, staphyloxanthin synthesis and adhesion associated genes in MRSA such as *sarA*, *crtM*, *icaA*, *icaD*, *fnbA*, *fnbB* and *altA*. Total RNA was isolated from hesperidin treated and an untreated sample by the Trizol method and cDNA was converted by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). As per the manufacturers protocol, the SYBR Green PCR master mix kit (Applied Biosystems) was used to perform qPCR analysis. After normalizing the housekeeping gene expression (*gyrB*), selected gene expressions were calculated using 2−ΔΔCt values (Chemmugil et al. 2019). The primer sequences of tested genes are listed in Table 1.

**Molecular docking study**

To evaluate the hesperidin antibiofilm activity against MRSA, molecular studies were done with SarA protein and CrtM protein which have been reported as major regulator mediated with biofilm formation and staphyloxanthin production in MRSA. Therefore, SarA (ID: 2FNP) and CrtM (ID: 2ZCO) crystal structure was retrieved from Protein Data Ban (PDB). Then, the 3D structure of target compound hesperidin (PubChem ID: 10,621) were obtained from PubChem database. Autodock Tools v1.5.6 was used to assess the interactions between hesperidin, SarA and CrtM of MRSA. Moreover, BIOVIA Discovery Studio visualizer 2016 v16.1.0.15350 was used to visualize docked complexes (Selvaraj et al. 2019).

**Statistics**

Each experiment was performed in triplicate to obtain statistical confidence. Data values of experimental results were recorded as the mean ± Standard Deviation. The significance was determined by using one way ANOVA as P-value < 0.05 (indicate*). Statistical analyses were performed using SPSS 20.0 statistical software.

**Results**

**Effect of hesperidin on biofilm formation of MRSA**

The ability of hesperidin to inhibit biofilm formation by MRSA was evaluated by CV staining biofilm quantification assay in 24 well polystyrene plates. The result showed a concentration-dependent antibiofilm potential of hesperidin against MRSA strains. Hesperidin at 100 µg/ml concentration showed a maximum inhibition of 91% (MRSA ATCC-33,591), 83% (MRSA-vk9) and 86% (MRSA-vk10) of biofilm inhibition in MRSA strains. (Fig. 1a, b, c). At above concentrations of 100 µg/ml didn’t exhibit significant biofilm inhibition. Hence, 100 µg/ml concentration was considered BIC and further studies were evaluated in this concentration.

**Microscopic analysis**

The antibiofilm potential of hesperidin was further clarified by light microscope and CLSM analysis. The light microscope showed a significant reduction of biofilm formation on glass slides (Fig. 2a). On the other hand, CLSM analysis showed a multi-layer of adherent cells along with micro and macro colonies formation in the MRSA control. In contrast, the MRSA biofilm formation in the 100 µg/ml of hesperidin showed a significant reduction of multi-layered adherent cells and reduced the surface colonization and biofilm thickness (Fig. 2b).

| Genes | 5′–3′ Forward primer | Reverse primer |
|-------|----------------------|----------------|
| sarA  | CAAAACACCACAAGTTGTTAAA | TGTFFGCTTCAGTGATTGTTT |
| icaA  | ACACITTGTGCCGAGCTCAAA | TCTGGAACCAACTCCAACA |
| icaD  | ATGGTCCAGCCAGACAGAG | AGTATTTCATGTTTTAAAGCA |
| altA  | TGGTACGATATTTGGCAGCTTGA | TGGATCTTCAACACTTCAAGAC |
| crtM  | ATCCGAACACCCCGTCTT | GCAGTGAAGGTATTGCGATT |
| fnbA  | ATCAGCGATGTATCCGGAAG | TTATTAGCCTGCCTGTTGCC |
| fnbB  | AAGAAGCGACGAAAACCTGTG | TCTCGAATGCGTGAACCC |
| gyrB  | GTGCTGGGCAATCAAAGT | TCCCAACTAAATGCTGCA |

Table 1 List of primers used for real-time PCR analysis
Effect of hesperidin on the growth of MRSA

A MRSA growth measurement was evaluated in the presence and absence of hesperidin. The result showed that hesperidin treatment could not affect MRSA growth (ATCC-33,591), clinical isolates of strains MRSAvk9 and MRSAvk10. Hesperidin treatment growth curve was similar to the control sample (Fig. 3a–c). Further, MRSA metabolic
activity was measured by XTT analysis. The resulting exhibit insignificant variation in hesperidin treated and untreated samples (Fig. 3d).

**Effect of hesperidin on autoaggregation and autolysin of MRSA**

MRSA biofilm formation has highly associated with autoaggregation and autolysin. Hence, the impact of hesperidin on autoaggregation and autolysin was assessed. In the autoaggregation assay, hesperidin showed a significant reduction in MRSA as compared to control (Fig. 4a). Note that hesperidin strongly inhibited the autolysin in MRSA whereas compared to the control sample (Fig. 4b).

**Effect of hesperidin on enzyme virulence factors of MRSA**

An enzyme is an important virulence factor in MRSA. The present study evaluated lipase and hemolysin production in clinical isolates. Error bars indicates SDs from the mean [n = 6 (biological triplicates in technical duplicates)]

![Fig. 3 Effect of hesperidin on growth and viability assessed by growth curve analysis and cell viability assay](image1)

(a) MRSA ATCC 33,591  
(b) MRSAvk9  
(c) MRSAvk10  
(d) cell viability assay of MRSA and their clinical isolates. Error bars indicates SDs from the mean [n = 6 (biological triplicates in technical duplicates)]

![Fig. 4 Effect of hesperidin on the autoaggregation of MRSA (a). Effect of hesperidin on the autolysin of MRSA (b). Error bars indicates SDs from the mean [n = 6 (biological triplicates in technical duplicates)]. *Indicate a statistically significant difference (p < 0.05)](image2)
The results showed that hesperidin significantly reduced the lipase and hemolysin production in MRSA compared to control samples (Fig. 5a, b).

**Effect of hesperidin on staphyloxanthin production in MRSA**

Staphyloxanthin is a golden-yellow colour carotenoid pigmentation that is responsible for regulating the antioxidant level in MRSA. Hence, the present study evaluated the staphyloxanthin production in hesperidin treatment. The result showed that hesperidin was strongly reduced the staphyloxanthin production in MRSA. In contrast, the control sample significantly synthesized staphyloxanthin production (Fig. 6a).

**Effect of hesperidin on the survival of MRSA in H₂O₂**

Hesperidin antistaphyloxanthin activity was evaluated, the effect of hesperidin on MRSA survival in an oxidant condition like H₂O₂. The result showed that hesperidin treatment significantly affected the survival of MRSA (6 × 10⁷) whereas compared to the control sample (1.8 × 10⁸) (Fig. 6b).

**Effect of hesperidin on the virulence genes expression of MRSA**

To evaluate the effect of hesperidin on MRSA virulence gene expression was assessed by RT-PCR. The result showed that hesperidin treatment downregulated the expression of biofilm associated, staphyloxanthin synthesis and adhesion associated genes such as sarA (2.2), crtM (2.1), icaA (1.8), icaD (2.3), altA (1.5), fnbA (0.9) and fnbB (0.5) (Fig. 7).

**Molecular docking analysis**

Molecular docking analysis revealed the ability of hesperidin to interact with SarA and CrtM proteins of MRSA. Hesperidin strongly interacts with active sites of SarA with the binding energy of −6.9 kcal/mol (Table 2) and exhibited three hydrogen bonding interactions (Thr 117 and Lys 163)
In the case of CrtM, hesperidin actively interacts through (Arg 158, Tyr 154 and Gln 102) (Fig. 8) and the binding energy of hesperidin with CrtM was −6.4 kcal/mol (Table 2).

**Discussion**

Biofilm-mediated infections by MRSA are much harder to treat by commercial antibiotics treatments; therefore, the emphasis on antibiofilm treatment-based work has been increasing in present times. Hesperidin is approved by the Food and Drug Administration (FDA) for use as a flavoring agent and a flavoring color in food industries and is also identified to have numerous biological potential. The present study proved that hesperidin inhibits biofilm formation and virulence production of MRSA (ATCC strain) and clinical isolates without any lethal effect on cell viability. Hesperidin inhibits biofilm formation of MRSA (ATCC-33,591) 91% and clinical isolates strains MRSA-vk9 (83%) and MRSA-vk10 (86%) at 100 µg/ml concentration (Fig. 1a–c) without affecting growth (Fig. 3a–c). The present study result was compared to previous study (Selvaraj et al. 2019) which reported that myrtenol (a flavonoid compound) significantly inhibited biofilm formation of MRSA ATCC strains and clinical isolates at 300 µg/ml. Hesperidin inhibitory concentration is lower than the myrtenol concentration, it might be added more value to pick the hesperidin as a potential therapeutic agent. Furthermore, light microscopy and CLSM images showed the significant inhibition of biofilm, microcolony formation and reduction of biofilm thickness upon hesperidin treatment (Fig. 2a, b). As the scope of the antibiofilm potential of hesperidin against clinical isolates was confirmed, therefore, MRSA ATCC strain alone was used for further experiments.

Autoaggregation has been reported as a key factor when biofilm formation occurred in MRSA (Houston et al. 2011). Notably, inoculation of hesperidin to the TSBS medium reduced the autoaggregation of MRSA in 100 µl/ml concentration (Fig. 4a). The non-toxic nature of hesperidin and its potential to inhibit biofilm formation and autoaggregation of MRSA which could be a good implication for drug development. Autolysis associated with eDNA is an essential factor in *S. aureus* for autoaggregation and biofilm formation. A previous study has been reported that autolysin (*AtlA*) mutants could affect biofilm formation (Houston et al. 2011). Here, the present study evaluates the hesperidin treatment on autolysin production in MRSA. It was analyzed spectrophotometrically. The results observed the occurrence of active lysis in the control cells, whereas treatment of hesperidin significantly affects the autolysis process in MRSA (Fig. 4b). The obtained results suggest that impairment of autoaggregation and autolysis process could be possible mechanisms responsible for control/preventing of biofilm formation.
Hesperidin also affects the lipase and α-hemolysin production in MRSA. These two virulence factors are responsible for break/digest the host tissue or lipids. S. aureus synthesis lipase production which is responsible for the accumulation of granulocytes, digest the host lipids and protects the cells from antimicrobial potent lipids secreted by human skin. The present study results showed that treatment of hesperidin at 100 µg/ml concentration reduced lipase production (Fig. 5b). Some pathogenic bacteria secrete pore forming toxins. The toxins contain water soluble monomeric proteins which settle on the membrane of target cells to form bilayer spanning pores (Iacovache et al. 2008). S. aureus secretes β-pore forming toxins (β-PFTs) such as α-Hemolysin, γ-Hemolysin, leuocidin and Panton-Valentine leukocidin (PVL). Among them, α-Hemolysin is an important virulence factor in S. aureus which is responsible for the necrosis of host cells. α-Hemolysin single polypeptide proteins are settled on the membrane of target cells, able to form a β-barrel transmembrane aqueous channel with a diameter of 14 Å and allow the K⁺ and Ca²⁺ ions into the target cells (Yamashita et al. 2011). Hence, the inhibition of α-hemolysin activity in S. aureus could be a possible strategy to control/prevent the host cell damage during infection. The results showed that treatment of hesperidin

Fig. 8 Molecular docking analysis. Representative images depicting the interactions of hesperidin with SarA and CrtM proteins in MRSA

| Receptor | Ligand | Key residue | Binding energy/docking score (kcal/mol) |
|----------|--------|-------------|----------------------------------------|
| SarA     | Hesperidin | Thr:117; Lys:163 | −6.9                                   |
| CrtM     | Hesperidin | Arg:158; Tyr:154; Gln:102 | −6.2                                   |

Table 2 Molecular docking analysis reveals hesperidin binding efficacy with SarA and CrtM of MRSA
at 100 μg/ml concentration inhibited hemolysin production (Fig. 5a). Notably, the auto-inducing peptide regulated the lipase and α-hemolysin production in *S. aureus*. The present findings were similar to the previous study; l-ascorbyl 2, 6-dipalmitate (ADP) treatment inhibits the autoinducing peptide regulated lipase and α-hemolysin production in *S. aureus* (Sethupathy et al. 2017). Reduction of lipase and α-hemolysin production by hesperidin proposed that it is capable of impeding auto-inducing peptide signaling in *S. aureus*.

The antioxidant property is required for *S. aureus* to survive in adverse in vivo environments and innate immune responses (Thammavongsa et al. 2015). Staphyloxanthin, a carotenoid pigment regulated the antioxidant level in *S. aureus*. A previous study has been reported that inhibition of staphyloxanthin pigment production leads to increasing the neutrophil-mediated killing and affects the *S. aureus* survival rate in an oxidative condition (Mani et al. 1993). Therefore, the inhibition of staphyloxanthin pigment production could be a positive approach to make *S. aureus* more susceptible to antibiotics and the host immune responses (Liu et al. 2012). Hesperidin inhibited staphyloxanthin production at 100 μg/ml concentration (Fig. 6a). Furthermore, we evaluated the effect of hesperidin treatment on the survival of MRSA in H₂O₂ oxidative stress. The results showed that the MRSA control cells significantly survive in H₂O₂ conditions. In contrast, hesperidin treated MRSA cells were highly susceptible to H₂O₂ (Fig. 6b). These results confirmed the potential of hesperidin to hamper staphyloxanthin production and significantly reduced the survival rate in H₂O₂ conditions. The present finding was compared with the previous study; rhodomyrtone treatment affects staphyloxanthin production as well as reduced the MRSA survival rate in the H₂O₂ environment (Leejae et al. 2013).

In this current situation, we need to find out an ideal antibiofilm/antipathogenic agent to control biofilm mediated pathogenic infections in clinical settings. In which bioactive components are only targeting pathogenic biofilm inhibition instead of inhibiting proliferative changes in metabolic growth of pathogens (Rasmussen and Givskov 2006). The growth curve analysis showed that the effect of hesperidin (100 μg/ml) doesn’t affect the MRSA metabolic growth and the kinetic growth measurements suggested that hesperidin was a non-bactericidal property in nature (Fig. 3a). Moreover, XTT assay results showed an insignificant difference in the metabolic growth of control and hesperidin treated MRSA at 100 μg/ml concentration (Fig. 3b).

To evaluate the effect of hesperidin on MRSA virulence gene expression was assessed by RT-PCR. Staphylococcal accessory regulator A (SarA) is a global virulence regulator in *S. aureus* and positively activates the biofilm formation during the inactivation of the accessory gene regulator (agr) system (Balamurugan et al. 2015; Li et al. 2016). Hence, the present study evaluates the effect of hesperidin on the sarA system. The results showed the downregulation of sarA upon hesperidin treatment (Fig. 7). The present result indicates the presumption that the antibiofilm potential of hesperidin could be sarA dependent. The intercellular adhesion locus (icaABCD) regulated the *S. aureus* cell to cell adhesion via the production of polysaccharides (Otto 2013). Then, the present study analyzed icaA (N-acetyl glucosamine) and icaD (poly-beta-1, 6-N-acetyl glucosamine biosynthesis protein) gene expression. Gene expression analysis revealed the downregulation of icaA and icaD upon hesperidin treatment (Fig. 7). A previous study reported that icaA gene is required for the action of icaD for significant production of polysaccharides in *S. aureus* (Cue et al. 2012). Hence, the downregulation of icaA by hesperidin could be responsible for the inhibition of biofilm formation in MRSA. Apart from ica locus, SarA controls some adhesion proteins such as fibronectin-binding proteins FnBA and FnBB (Li et al. 2019; Kot et al. 2019). Hesperidin treatment downregulated the sarA and sarA associated fnbA and fnbB genes (Fig. 7). A similar expression profile was observed in the previous study, myrtenol treatment reduced the expression of sarA and their associated fnbA and fnbB (Selvaraj et al. 2019). Moreover, hesperidin treatment significantly reduced the autolysis in MRSA. Real-time PCR analysis confirms the autolysin inhibitory potential of hesperidin. Hesperidin treatment significantly downregulated the altA gene expression in MRSA (Fig. 7). The present finding was similar to the previous study, myrtenol affects the autolysin by downregulation of altA in MRSA (Selvaraj et al. 2019). Downregulation of these genes responsible for biofilm formation by hesperidin could be a promising mechanism responsible for its antibiofilm potential. *crtM* is an important enzyme responsible for the synthesis of staphyloxanthin in *S. aureus* (Pelz et al. 2005). The downregulation of *crtM* was observed in hesperidin treatment (Fig. 7) which could be a possible reason for staphyloxanthin inhibition in MRSA. The present finding was similar to the previous study, 5-dodecanolide downregulated the *crtM* in MRSA (Valliammai et al. 2019).

In modern days, structure-based virtual screening methods help with the identification and design of a wide range of bioactive compounds against drug targets. In silico, an approach makes it easier to forecast the interactions of recognized drug target proteins and their native ligands (Kahlon et al. 2010). In the present study, we evaluate the active binding site of hesperidin on sarA and crtM in MRSA. The results showed that hesperidin interacts with the active site of SarA (Thr 117 and Lys 163) (Fig. 8) and the binding activity was recorded in Table 2. On the other hand, hesperidin interacts with the active site of CrtM (Arg 158, Tyr 154 and Gln 102) (Fig. 8) and the binding activity was recorded in Table 2. These results suggest that the binding of hesperidin to the target sites of active enzymes could be a
possible mechanism responsible for the inhibition of biofilm formation and staphyloxanthin production in MRSA.

In conclusion, the present study evaluated the antibiofilm potential of hesperidin against MRSA. Hesperidin revealed strong antibiofilm potential against MRSA (ATCC-33,591), clinical isolates MRSAvk9 AND MRSAvk10 without affecting metabolic growth. Hesperidin inhibited autolysis, auto-aggregation, lipase and hemolysin production in MRSA. qPCR analysis revealed the downregulation of virulence genes involved in biofilm formation and virulence factor production in MRSA upon hesperidin treatment. Moreover, molecular docking analysis predicted the ability of hesperidin to bind the active sites of SarA and CrtM in MRSA. This pilot study suggests that hesperidin could be a potential anti-biofilm and antivirulence agent against MRSA pathogenesis.

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Conflict of interest All the authors declare that they have no conflict of interest.

Ethical approval This study does not contain any experiments with human participants or animals performed by any of the authors.

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