Hesperidin inhibits biofilm formation, virulence and staphyloxanthin synthesis in methicillin resistant *Staphylococcus aureus* by targeting SarA and CrtM: An *in vitro* and *in silico* approach

Vijayakumar Karuppiah (✉ vijay92biotech@gmail.com)

Annamalai University Faculty of Marine Sciences <https://orcid.org/0000-0002-7651-760X>

Muhilvannan Seralathan

PAR life sciences and research laboratory

Arunvignesh Mani

PAR LIFE SCIENCE AND RESEARCH LABORATORY

Research Article

Keywords: Hesperidin, *Staphylococcus aureus*, SarA, CrtM, Antibiofilm

Posted Date: October 7th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-911921/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Methicillin resistant *Staphylococcus aureus* is considered the 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 *S. aureus*. In addition, hesperidin treatment significantly impedes lipase, hemolysin, autolysin, autoaggregation and staphyloxanthin production. Reductions of staphyloxanthin production possibly increase the MRSA susceptibility rate to H₂O₂ oxidative stress condition. In gene expression study revealed the 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.

Introduction

Methicillin resistant *Saphylococcus 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 by 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; WHO 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 factors 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 it acts 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 failure 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, such as 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; Vijayakumar and Ramanathan 2020; Karuppiah and Thirunanasambandham 2020; Vijayakumar et al. 2020a; Vijayakumar et al. 2020b; 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. The present study mainly focused on screening bioactive components with antibiofilm potential against MRSA.

Hesperidin is a plant based bioactive component 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 anticancer 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 Staphylococcus aureus (ATCC 33591) was purchased from ATCC, Himedia, India and clinical isolate strains MRSAvk9 (GenBank accession number MK757651) and MRSAvk10 (GenBank accession number MK757652) was 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 was performed in 24 well polystyrene plate as performed earlier (Karuppiah and

Thirunanasambandham 2020). Briefly, 1 % of MRSA culture 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 without hesperidin and 2 ml of TSBS 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-33591), clinical isolates MRSAvk9 and MRSAvk10 were inoculated into 1 ml of TSBS medium in the 24 well plate, inserting 1 X 1cm 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 400X under the light microscope. In confocal laser scanning microscope (CLSM), slides were stained with 0.1 % of acridine orange solution and observed at 200X in CLSM (Karuppiah and Thirunanasambandham 2020).

Growth curve analysis

To evaluate the effect of hesperidin on the growth of MRSA (ATCC-33591), clinical isolates MRSAvk9 and MRSAvk10, 100 ml of TSBS broth was inoculated with 1 % inoculum of MRSA (1.6×10^7 CFU ml⁻¹) 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 MRSA were quantified using the XTT (2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-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 assayed using the method. 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 50mM Na₂PO₄ buffer (pH 7.0) containing 0.2 % sodium deoxycholate and 0.1 % gumini arabicum and incubated at room temperature in dark for 1h. Following incubation, lipase activity was terminated by adding equal volume of 1M 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 (Sethupathy et al. 2017).

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 BHI medium to a final concentration of 2 % (v/v) and divided into three aliquots. The first aliquot, 10 % 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. Briefly, MRSA was grown in 100 ml of TSBS along with hesperidin 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 (Leejae et al. 2013).

H₂O₂ sensitivity assay

To determine the sensitivity of MRSA cells on H₂O₂ in the presence and absence of 5HMF the method was followed described earlier (Valliammai et al. 2019). Briefly, control and hesperidin treated cell suspensions were centrifuged at 8000 x 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 $2^{-\Delta\Delta Ct}$ 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 Bank (PDB). Then, the 3D structure of target compound hesperidin (PubChem ID: 10621) 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 $\pm$ 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-33591), 83 % (MRSA-vk9) and 86 % (MRSA-vk10) of biofilm inhibition in MRSA strains. (**Fig. 1a, b, c**). At above concentrations of 100 µg/ml doesn'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**).

Effect of hesperidin on the growth of MRSA

A MRSA's growth measurement was evaluated in the presence and absence of hesperidin. The result showed that hesperidin treatment could not affect MRSA growth (ATCC-33591), clinical isolates of strains MRSAvk9 and MRSAvk10. Hesperidin treatment growth curve was similar to the control sample (**Fig. 3a-c**). Further, MRSA's 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 was 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 hesperidin treatment. The results showed that hesperidin was significantly reduced the lipase and hemolysin production in MRSA whereas 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 was 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 (6×10^7) whereas compared to the control sample (1.8×10^8) (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) (Fig. 8). 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-33591) 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, leucocidin 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 Ca^{2+} 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 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 impede auto-inducing peptide signaling in *S. aureus*.

The antioxidant property is required for *S. aureus* to survival in adverse in vivo environments and innate immune responses (Thammavongsa et al. 2015). Staphyloxanthin, a carotenoid pigmentation regulated the antioxidant level in *S. aureus*. The 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. The zaragozic acid and rhodomyrtone have been reported to have significant inhibitory activity against staphyloxanthin pigment production in MRSA (Leejae et al. 2013; 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 have to need 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 was 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, 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.

The present study evaluated the antibiofilm potential of hesperidin against MRSA. Hesperidin revealed strong antibiofilm potential against MRSA (ATCC-33591), clinical isolates MRSAvk9 AND MRSAvk10 without affecting metabolic growth. Hesperidin inhibited autolysin, autoaggregation, 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 antibiofilm and antivirulence agent against MRSA pathogenesis.

Declarations

Acknowledgements

The authors gratefully thank Dr. G. Ashwinkumar Subramenium for support in discussing and fulfilling the work. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Funding

Not applicable

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

Conflict of interest

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

Authors Contribution list:

KV conceived and designed the research, conducted experiments and wrote the manuscript. SM contributed to analyzed data. MA conducted the molecular docking analysis.

ORCID

KarupiahVijayakumar <https://orcid.org/0000-0002-7651-760x>

References

1. Troeman DPR, Van Hout D, Kluytmans JAJW (2019) Antimicrobial approaches in the prevention of *Staphylococcus aureus* infections: a review. J Antimicrob Chemother 74:281-294.
2. Lee AS, de Lencastre H, Garau J, Kluytmans J, Malhotra-Kumar S, Peschel A, Harbarth S (2018) Methicillin-resistant *Staphylococcus aureus*. Nat Rev Dis Primers 4:1-23.
3. Antibiotic resistance: Multi-country public awareness survey I. World Health Organization. ISBN 978 92 4 150981 7 Subject headings are available from WHO institutional repository. 2015 [cited 2017 June]. Available from: http://apps.who.int/iris/bitstream/10665/194460/1/9789241509817_eng.pdf?ua=1.
4. WHO. Media Centre. News Release. WHO publishes list of bacteria for which new antibiotics are urgently needed. 2017 [cited 2017 March]. Available from: <http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/>.
5. Jenul C, Horswill AR (2019) Regulation of *Staphylococcus aureus* virulence. Microbiol Spectr 7:7-2.
6. Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O (2010) Antibiotic resistance of bacterial biofilms. Int J Antimicrob Agents 35:322-332.
7. Stewart PS, Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. The Lancet 358:135-138.
8. Liu Q, Yeo WS, Bae T (2016) The SaeRS two-component system of *Staphylococcus aureus*. Genes 7:81.
9. Tan L, Li SR, Jiang B, Hu XM, Li S (2018) Therapeutic targeting of the *Staphylococcus aureus* accessory gene regulator (agr) system. Front Microbiol 9:55.
10. Clauditz A, Resch A, Wieland KP, Peschel A, Götz F (2006) Staphyloxanthin plays a role in the fitness of *Staphylococcus aureus* and its ability to cope with oxidative stress. Infect Immun 74:4950-4953.
11. Otto M (2013) Staphylococcal infections: mechanisms of biofilm maturation and detachment as critical determinants of pathogenicity. Annu Rev Med 64:175-188.

12. Roy R, Tiwari M, Donelli G, Tiwari V (2018) Strategies for combating bacterial biofilms: a focus on anti-biofilm agents and their mechanisms of action. *Virulence* 9:522–554.
13. Mishra R, Panda AK, De Mandal S, Shakeel M, Bisht SS, Khan J (2020) Natural anti-biofilm agents: Strategies to control biofilm-forming pathogens. *Front Microbiol* 11:2640.
14. Rabin N, Zheng Y, Opoku-Temeng C, Du Y, Bonsu E, Sintim HO (2015) Agents that inhibit bacterial biofilm formation. *Future Med Chem* 7:647– 671.
15. Atanasov AG, Zotchev SB, Dirsch VM, Supuran CT (2021) Natural products in drug discovery: Advances and opportunities. *Nat Rev Drug Discov* 20:200-216.
16. Subramenium GA, Vijayakumar K, Pandian SK (2015) Limonene inhibits streptococcal biofilm formation by targeting surface-associated virulence factors. *J Med Microbiol* 64:879-890.
17. Vijayakumar K, Ramanathan T (2018) Antiquorum sensing and biofilm potential of 5-Hydroxymethylfurfural against Gram positive pathogens. *Microb Pathog* 125:48-50.
18. Vijayakumar K, Ramanathan T (2020) *Musa acuminata* and its bioactive metabolite 5-Hydroxymethylfurfural mitigates quorum sensing (las and rhl) mediated biofilm and virulence production of nosocomial pathogen *Pseudomonas aeruginosa* in vitro. *J Ethnopharmacol* 246:112242.
19. Karuppiiah V, Thirunanasambandham R (2020) Quebrachitol from *Rhizophora mucronata* inhibits biofilm formation and virulence production in *Staphylococcus epidermidis* by impairment of initial attachment and intercellular adhesion. *Archiv Microbiol* 202:1327-1340.
20. Vijayakumar K, Bharathidasan V, Manigandan V, Jeyapragash D (2020) Quebrachitol inhibits biofilm formation and virulence production against methicillin-resistant *Staphylococcus aureus*. *Microb Pathog* 149:104286.
21. Vijayakumar K, Manigandan V, Jeyapragash D, Bharathidasan V, Anandharaj B, Sathya M (2020) Eucalyptol inhibits biofilm formation of *Streptococcus pyogenes* and its mediated virulence factors. *J Med Microbiol* 69:1308-1318.
22. Vijayakumar K, Thirunanasambandham R (2021) 5-Hydroxymethylfurfural inhibits *Acinetobacter baumannii* biofilms: an in vitro study. *Archiv Microbiol* 203:673-682.
23. Karuppiiah V, Thirunanasambandham R, Thangaraj G (2021) Anti-quorum sensing and antibiofilm potential of 1, 8-cineole derived from *Musa paradisiaca* against *Pseudomonas aeruginosa* strain PAO1. *World J Microbiol Biotechnol* 37:1-12.
24. Corciova A, Ciobanu C, Poiata A, Mircea C, Nicolescu A, Drobeta M, Varganici CD, Pinteala T, Marangoci N (2015) Antibacterial and antioxidant properties of hesperidin: β -cyclodextrin complexes obtained by different techniques. *J Incl Phenom Macrocycl Chem* 81:71-84.
25. Samie A, Sedaghat R, Baluchnejadmojarad T, Roghani M (2018) Hesperetin, a citrus flavonoid, attenuates testicular damage in diabetic rats via inhibition of oxidative stress, inflammation, and apoptosis. *Life Sci* 210:132-139.
26. Sorroche FG, Spesia MB, Zorreguieta A, Giordano W (2012) A positive correlation between bacterial autoaggregation and biofilm formation in native *Sinorhizobium meliloti* isolates from Argentina. *Appl*

27. Cue D, Junecko JM, Lei MG, Blevins JS, Smeltzer MS, Lee CY (2015) SaeRS-dependent inhibition of biofilm formation in *Staphylococcus aureus* Newman. PLoS One 10:e0123027.
28. Sethupathy S, Vigneshwari L, Valliammai A, Balamurugan K, Pandian SK (2017) L-Ascorbyl 2, 6-dipalmitate inhibits biofilm formation and virulence in methicillin-resistant *Staphylococcus aureus* and prevents triacylglyceride accumulation in *Caenorhabditis elegans*. RSC Adv 7:23392-23406.
29. Hollands A, Aziz RK, Kansal R, Kotb M, Nizet V, Walker MJ (2008) A naturally occurring mutation in *ropB* suppresses SpeB expression and reduces M1T1 group A streptococcal systemic virulence. PLoS One 3:e4102.
30. Leejae S, Hasap L, Voravuthikunchai SP (2013) Inhibition of staphyloxanthin biosynthesis in *Staphylococcus aureus* by rhodomyrtone, a novel antibiotic candidate. J Med Microbiol 62:421-428.
31. Valliammai A, Sethupathy S, Priya A, Selvaraj A, Bhaskar JP, Krishnan V, Pandian SK (2019) 5-Dodecanolide interferes with biofilm formation and reduces the virulence of Methicillin-resistant *Staphylococcus aureus* (MRSA) through up regulation of agr system. Sci Rep 9:1-16.
32. Chemmugil P, Lakshmi PTV, Annamalai A (2019) Exploring Morin as an anti-quorum sensing agent (anti-QSA) against resistant strains of *Staphylococcus aureus*. Microb Pathog 127:304-315.
33. Selvaraj A, Jayasree T, Valliammai A, Pandian SK (2019) Myrtenol attenuates MRSA biofilm and virulence by suppressing sarA expression dynamism. Front Microbiol 10:2027.
34. Houston P, Rowe SE, Pozzi C, Waters EM, O'Gara JP (2011) Essential role for the major autolysin in the fibronectin-binding protein-mediated *Staphylococcus aureus* biofilm phenotype. Infect Immun 79:1153-1165.
35. Iacovache I, van der Goot FG, Pernot L (2008) Pore formation: an ancient yet complex form of attack. Biochem Biophys Acta Biomembr 1778:1611-1623.
36. Yamashita K, Kawai Y, Tanaka Y, Hirano N, Kaneko J, Tomita N, Ohta M, Kamio Y, Yao M, Tanaka I (2011) Crystal structure of the octameric pore of staphylococcal γ -hemolysin reveals the β -barrel pore formation mechanism by two components. Proc Natl Acad Sci 108:17314-17319.
37. Thammavongsa V, Kim HK, Missiakas D, Schneewind O (2015) Staphylococcal manipulation of host immune responses. Nat Rev Microbiol 13:529-543.
38. Mani N, Tobin P, Jayaswal RK (1993) Isolation and characterization of autolysis-defective mutants of *Staphylococcus aureus* created by Tn917-lacZ mutagenesis. J Bacteriol 175:1493-1499.
39. Liu CI, Jeng WY, Chang WJ, Ko TP, Wang AHJ (2012) Binding modes of zaragozic acid A to human squalene synthase and staphylococcal dehydrosqualene synthase. J Biol Chem 287:18750-18757.
40. Rasmussen TB, Givskov M (2006) Quorum sensing inhibitors: a bargain of effects. Microbiology 152:895-904.
41. Balamurugan P, Hema M, Kaur G, Sridharan V, Prabu PC, Sumana MN, Princy SA (2015) Development of a biofilm inhibitor molecule against multidrug resistant *Staphylococcus aureus* associated with gestational urinary tract infections. Front Microbiol 6:832.

42. Li L, Cheung A, Bayer AS, Chen L, Abdelhady W, Kreiswirth BN, Yeaman MR, Xiong YQ (2016) The global regulon *sarA* regulates β -lactam antibiotic resistance in methicillin-resistant *Staphylococcus aureus* in vitro and in endovascular infections. J Infect Dis 214:1421-1429.
43. Cue DR, Lei MG, Lee C (2012) Genetic regulation of the intercellular adhesion locus in staphylococci. Front Cell Infect Microbiol 2:38.
44. Li X, Huang T, Xu K, Li C, Li Y (2019) Molecular characteristics and virulence gene profiles of *Staphylococcus aureus* isolates in Hainan, China. BMC Infect Dis 19:1-12.
45. Kot B, Sytykiewicz H, Sprawka I, Witeska M (2019) Effect of trans-cinnamaldehyde on methicillin-resistant *Staphylococcus aureus* biofilm formation: Metabolic activity assessment and analysis of the biofilm-associated genes expression. Int J Mol Sci 21:102.
46. Pelz A, Wieland KP, Putzbach K, P. Hentschel, Albert K, Götz F (2005) Structure and biosynthesis of staphyloxanthin from *Staphylococcus aureus*. J Biol Chem 280:32493-32498.
47. Kahlon AK, Roy S, Sharma A (2010) Molecular docking studies to map the binding site of squalene synthase inhibitors on dehydrosqualene synthase of *Staphylococcus aureus*. J Biomol Struct Dyn 28:201-210.

Tables

Table. 1 List of primers used for real-time PCR analysis.

Genes	Forward primer 5' - 3'	Reverse primer 5' - 3'
<i>sarA</i>	CAAACAACCACAAGTTGTAAA	TGTTTGCTTCAGTGATTCGTTT
<i>icaA</i>	ACACTTGCTGGCGCAGTCAA	TCTGGAACCAACATCCAACA
<i>icaD</i>	ATGGGTCAGCCCAGACAGAG	AGTATTTTCAATGTTTAAAGCA
<i>altA</i>	TGTCGAAGTATTTGCCGACTTCG	TGGAATCCTGCACATCCAGGAAC
<i>crtM</i>	ATCCAGAACCACCCGTTTT	GCGATGAAGGTATTGGCATT
<i>fnbA</i>	ATCAGCAGATGTAGCGGAAG	TTTAGTACCGCTCGTTGTCC
<i>fnbB</i>	AAGAAGCACCGAAAACGTGTG	TCTCTGCAACTGCTGTAACG
<i>gyrB</i>	GGTGCTGGGCAAATACAAGT	TCCCACACTAAATGCTGCAA

Table. 2 Molecular docking analysis reveals hesperidin binding efficacy with SarA and CrtM of 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

Figures

Figure 1

Antibiofilm activity of hesperidin against MRSA ATCC-33591 and clinical isolates MRSAvk9 and MRSAvk10 strain. A 24-well microtitre plate depicting concentration dependent biofilm inhibition in MRSA. Percentage of biofilm inhibition by hesperidin assessed by crystal violet quantification assay. Error bars indicates SDs from the mean [n = 6 (biological triplicates in technical duplicates)]. * indicate a statistically significant difference (p < 0.05).

Figure 2

(a) Light microscope and (b) CLSM observation of MRSA ATCC-33591 and clinical isolates MRSAvk9 and MRSAvk10 strain biofilms formed on glass surfaces in the presence and absence of hesperidin.

Figure 3

Effect of hesperidin on the growth (a) and cell viability activity (b) of MRSA. Error bars indicates SDs from the mean [n = 6 (biological triplicates in technical duplicates)].

Figure 4

Effect of hesperidin on the autoaggregation of MRSA (a). Effect of hesperidin on the autolysis 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).

Figure 5

Hesperidin inhibits the production hemolysin (a) and lipase in biofilm inhibitory concentration. Error bars indicates SDs from the mean [n = 6 (biological triplicates in technical duplicates)].

Figure 6

Anticarotenogenic potential of hesperidin against MRSA. Effect of hesperidin treatment inhibits the staphyloxanthin production of MRSA (a). Hesperidin treatment affects the ability of MRSA to survive in the presence and absence of H2O2 (b). Error bars indicates SDs from the mean [n = 6 (biological triplicates in technical duplicates)].

Figure 7

Effect of hesperidin treatment on the gene expression of MRSA. Hesperidin treatment downregulated the expression of genes responsible for biofilm formation and virulence factor production in MRSA. Error bars indicates SDs from the mean [n = 6 (biological triplicates in technical duplicates)]. * indicate a statistically significant difference (p < 0.05).

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