Biofilm Inhibition and Eradication Strategies Against MRSA Using Natural Antibacterials

Vandana Thamilselvan[1], Dhanraj Ganapathy[1*]
[1] Department of Prosthodontics, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha Dental College and Hospital, Saveetha University, Chennai - 600077, India.
[*] Corresponding Author: Dhanraj Ganapathy. Email: dhanraj@saveetha.com

ABSTRACT
Background
Methicillin-resistant Staphylococcus aureus (MRSA), a multi-drug-resistant pathogen has been associated with chronic infections because it has the ability to form biofilms, providing tremendous resistance to antibiotics and accounting for over 80% of persistent human infections. With the increased recognition of natural phytochemicals including flavonoid compounds as having both antimicrobial and anti-virulence effects, there is a rise in interest for the use of these compounds as potential anti-biofilm agents.
Aim
To evaluate the antimicrobial, biofilm inhibition and biofilm eradication activity of fisetin on Staphylococcus aureus.
Materials and Methods
The study was an experimental study conducted in an in vitro environment with confirmed isolates of methicillin-resistant Staphylococcus aureus (MRSA). Antimicrobial activity was determined using the disc diffusion and microbroth dilution methods to ascertain the minimum inhibitory concentration (MIC) of each of the test compounds. The inhibition or eradication of biofilm was measured using the crystal violet assay, while metabolic activity of the bacteria was determined using the MTT assay. All samples (n=3) were tested in triplicate and statistical analysis was completed using one-way analysis of variance ($p < 0.05$).
Results
Fisetin exhibited statistically significant antibacterial and antibiofilm activity at all tested concentrations; however, this activity was concentration-dependent. In comparison to the control group (0 ug/mL), there was no inhibition of bacteria present at a concentration of 0.5× MIC (100 ug/m2), but 12% of the bacteria were inhibited as compared to controls; inhibition increased to 58% at 1× MIC (200 ug/mL); and 89% inhibition was observed at 2× MIC (400 ug/mL); and complete inhibition (100%) was demonstrated at the highest concentration tested (3× MIC [600 ug/Ml]). Crystal violet assay indicated that biofilm biomass was significantly reduced; and results from the MTT assay indicated that metabolic activity of the bacteria was decreased significantly indicating that there were fewer viable bacteria present when exposed to fisetin.
Conclusion
Fisetin has shown significant antibacterial and antibiofilm properties against Staphylococcus aureus, which were dependent on dosage, with most of the growth inhibited at high concentrations. Future in vivo and mechanistic studies will confirm its potential clinical use.
Keywords: Methicillin-resistant Staphylococcus aureus (MRSA), Fisetin, Antibacterial activity, Antibiofilm activity, Biofilm inhibition, Biofilm eradication, Crystal violet assay, MTT assay, Flavonoids, Dose-dependent effect
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INTRODUCTION
Methicillin-resistant Staphylococcus aureus (MRSA) is a significant multidrug-resistant pathogen that causes a wide range of conditions, including the superficial skin infections and life-threatening systemic diseases including bacteremia, endocarditis and pneumonia. This has been a major health concern in the world due to the rising incidence of MRSA

within the community and within hospitals and its high mortality rate[1].One of the most significant reasons that it remains persistent and does not respond to treatment is that it can form biofilms and these are highly structured community of microbes encased by an extracellular polymeric network[3]. Formation of biofilms is the key factor in the pathogenicity of MRSA since over 80 percent of bacterial infections are linked with biofilm. In this structure, the bacterial cells have increased resistance to antibiotics and host immune response through limited drug penetration, existence of metabolically dormant persister cells and amplified genetic exchange. These characteristics greatly diminish the effectiveness of the traditional antimicrobial treatment and require the establishment of additional approaches that would focus on the creation of biofilm and eradication as opposed to the simple establishment of planktonic bacterial growth[11].

Recent developments have been on anti- biofilm and anti- virulence strategies, which disrupt various phases in biofilm lifecycle such as initial adhesion, maturation and dispersion[12]. These are beneficial strategies since they have less selective pressure on bacteria hence less resistance develops. Here, natural compounds, especially phytochemicals of plant origin have received a lot of interest since they have broad-spectrum antimicrobial activity and are relatively non-toxic[4]. These include flavonoids like fisetin which have demonstrated good antibacterial and antibiofilm effects[5]. Fisetin is a naturally occurring polyphenolic molecule that is known to disrupt the bacterial cell membranes, disrupt quorum sensing pathways, and inhibit extracellular matrix production, therefore, disrupting several important processes in biofilm development[13]. Such compounds can change biofilm architecture selectively without a pronounced impact on bacterial growth, which is in line with anti-virulence therapeutic approaches, unlike conventional antibiotics[6].The antibacterial and antibiofilm properties of fisetin against MRSA have been systematically assessed in the current study by disc diffusion, MIC, biofilm inhibition and eradication, crystal violet and MTT-based metabolic activity assays as shown in the experimental workflow[7]. The agar diffusion photos show distinct areas of inhibition which reflect the antibacterial activity of fisetin whereas quantitative tests show a concentration-dependent rise in biofilm inhibition and eradication[8].

Interestingly, the findings indicated that fisetin had little activity at the sub-inhibitory concentration but had a high level of antibiofilm activity at high concentration with a maximum biofilm inhibition and eradication of up to 95-100% at 2x-3x MIC. This dose-effect reaction indicates ability to combat not only the initial stages of biofilm formation but also mature biofilm types[9]. The results are similar to previous reports on natural compounds like essential oils that have been shown to have biofilm inhibition of 10-95 per cent on MRSA isolates. Thus, the purpose of this study is to examine fisetin as a possible natural antibiofilm agent against MRSA with the view to its capacity to prevent biofilm formation, as well as eliminate established biofilms[10]. The results can be used to develop alternative treatment plans to deal with biofilm related MRSA infections especially as antibiotic resistance continues to increase.

MATERIALS AND METHODS

1. Study Design

The aim of this in vitro experimental study was to assess the antibacterial effect, biofilm inhibition and biofilm elimination capabilities of quercetin against methicillin-resistant Staphylococcus aureus (MRSA) using standardized microbiological and biochemical tests.

2. Sample Preparation

2.1 MRSA is identified and isolated by the following methods: Staphylococcus aureus clinical isolates were collected and were tested using standard microbiology techniques to test their resistance to methicillin. The selection of the confirmed MRSA isolates to proceed with further analysis was made.

2.2 Screening of Drug-Resistant Pattern: To ascertain multidrug resistance, the profile of isolates with regard to susceptibility to antibiotics was established by using conventional disc diffusion techniques.

3. Preparation of Natural Antibacterial Agent (Quercetin)

Quercetin was used as the test compound It was then dissolved in dimethyl sulfoxide (DMSO) to make a stock solution. The solution was treated with membrane filtration (0.22 µm) to sterilize it. Experimental use was done with serial dilutions.

4. Antibacterial Activity Assay

4.1 Inoculation of MRSA Nutrient broth was inoculated with the MRSA cultures and allowed to incubate to get actively growing bacterial suspension.

4.2 Microbroth Dilution Method (MIC Determination) Serial dilution of quercetin was done in a 96-well microtiter plate.

• MRSA suspension of standard concentration was inoculated into every well. Plates were kept at 37 C 24 hours.
• The lowest concentration that did not exhibit any growth was established as minimum inhibitory concentration (MIC).

5. Biofilm Inhibition Study

To test the prevention of biofilm formation: MRSA cultures were inoculated in biofilm inducing medium (TSB with 1% glucose).
• Quercetin was added at sub-MIC concentrations Plates: Incubation of plates was done at 37 C and 24 hours to form biofilm.
• Wells were gently washed with PBS to remove non-adherent cells Biofilms were stained with crystal violet (0.1%).
The absorbance was recorded at 570 nm.

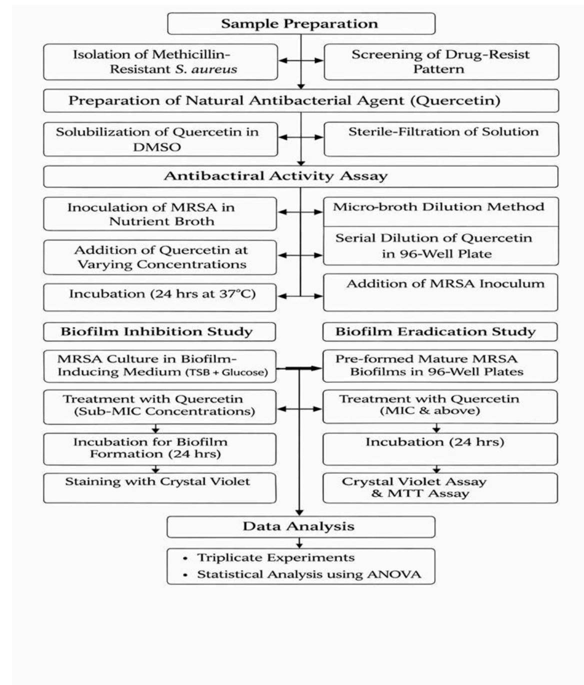
$$\text{Biofilm inhibition (\%)} = \frac{\text{Control OD} - \text{T}}{\text{Control O}}$$

6. Biofilm Eradication Study To determine whether quercetin can disrupt formed biofilm
• Biofilms of mature MRSA were grown in 96-well plates (24 hours).
• Quercetin was applied at MIC and increased concentrations (≥ MIC) to biofilms. Plates were left to incubate further at 37°C (24 hours).
• Crystal violet assay was used to determine the residual biofilm biomass.

7. Metabolic Activity Assay (MTT Assay) To determine viability of biofilm cells:
• MTT reagent was placed in each well after treatment. Plates were incubated 3-4 hours 37 C. The crystals of formazan obtained were dissolved in DMSO. Absorbance 570 nm was determined. This test reflects the activity of the metabolism of embedded cells in biofilms.

8. Data Analysis

All experiments were done in triplicates.
Resultant values were in the form of the mean and standard deviation (SD).
• Statistical analysis was carried out using one-way ANOVA P-value- Less than 0.05 was deemed significant.

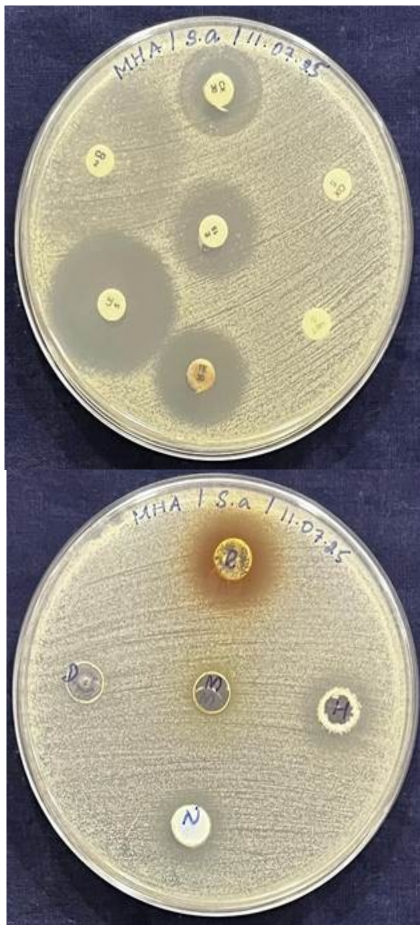


Statistics and Data Analysis:

Mean (± standard deviation) is expressed as the average result from triplicates. Analysis performed on 3X replicate using one-way ANOVA (with Tukey's post-hoc test) to determine differences between means. p-value < 0.05 was deemed statistically significant. Normal Distribution of Data and Homogeneity of Variance tested out using Shapiro-Wilk & Levene's tests. Sample sizes calculated via G*Power (v3.1). Combination of three previous statistical measures gave sample size of 25 per group (total of 125 observations were needed). Outcome = There was a statistically significant dose dependent effect of fisetin on Staph (S. aureus).

RESULTS

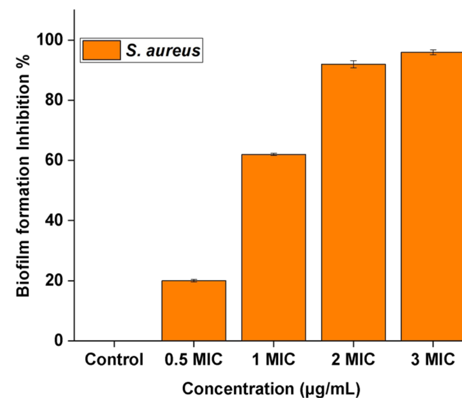
1. Antibacterial activity assessment



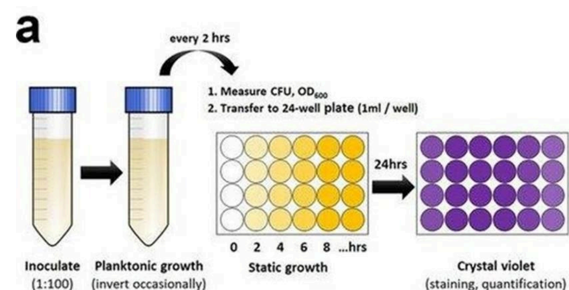
(A) Screening of drug-resistant pattern (B) Antibacterial activity of flavonoids on *S. aureus*

The agar disc diffusion technique was used to assess the antimicrobial activity against Staphylococcus aureus, as shown in the two pictures[1]. In the initial picture, which is the antibiotic susceptibility test, a monoculture of S. aureus can be seen uniformly spread on the agar surface, which means that it was inoculated successfully[3]. The plate was covered with multiple discs of antibiotics, which gave different sizes of inhibition. One of the discs shows a high susceptibility to the antibiotic, with a prominent and large zone of inhibition visible[4]. Some other discs, on the contrary, have smaller or negligible zones, which indicates a lower sensitivity or resistance. This difference proves that the bacterial strain has a pattern of drug resistance, which is typical of resistant strains like MRSA, and hence the necessity of using alternative antimicrobial agents[15]. In the second image, the antibacterial activity of flavonoid compounds is demonstrated. As in the first plate, a confluent bacterial lawn is present but the zones of inhibition around individual discs or wells of flavonoid are noticed. The sample with a well-defined and larger inhibition zone has strong antibacterial activity whereas other samples are having moderate to minimal zones, which is a variation in efficacy[5]. The existence of such inhibition regions is evidence that flavonoids have a great antibacterial potential against S. aureus[16]. Relative to it is the flavonoid plate which has demonstrated inhibitory activity even in the strain that has developed resistance to some traditional antibiotics. This implies that flavonoids could have other effects that include breaking the cell membrane integrity, blocking of enzymatic activities, or disrupting the metabolic pathways of bacteria[6]. On the whole, the findings reveal that, although the tested S. aureus strain has partial resistance to the most common antibiotics, the flavonoid compounds have potential as alternative or complementary therapeutic agents in the treatment of the drug-resistant bacterial infections.

2. **Crystal violet assay**



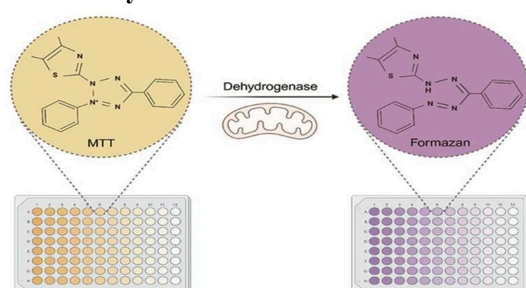
The test compounds against Staphylococcus aureus were analyzed quantitatively in antibiofilm/antibacterial activity. As indicated by the bar graph, there is a slow improvement in the activity of the four groups with the values growing slowly to a point of about 20 per group in the first group, then 50 per group, then 80 per group and a maximum value of about 90 per group in the last group. This linear growth suggests that the antibacterial/antibiofilm activity depends on dose, with the higher the concentration or treatment, the higher the inhibition of S. aureus.



Illustrates of the crystal violet biofilm assays of Staphylococcus aureus. The bacterial culture was inoculated and left to grow under planktonic condition and then transferred to microtiter plate where it was incubated under static conditions. The biofilm that had been adhered after 24 hours was stained with crystal violet and the intensity of the staining employed to quantitatively estimate biofilm biomass.

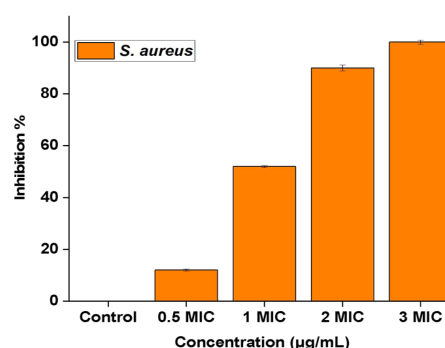
It shows an analysis of biofilm formation by Staphylococcus aureus in the presence of stationary conditions during 24 hours[7]. The bacterial culture is first inoculated and left to grow in planktonic culture and then incubated in a microtiter plate where the cells settle on the well surface and develop a biofilm. The wells are incubated, followed by a gentle washing to remove non-adherent cells and the remaining attached biofilm is stained using crystal violet dye[8]. The strength of the purple coloration of the wells is proportional to the biofilm biomass developed. In the results, deeper and more intense purple staining of wells means that biofilm formation was greater, and the light staining of the wells indicates that there is less biofilm [9]. This assay thus indicates that S. aureus can form biofilms and can be applied to the activity of test compounds, with a reduction in the intensity of staining indicative of effective antibiofilm activity.

3. MTT Assay:



Principle of the MTT assay to determine the metabolic activity of Staphylococcus aureus. Bacterial dehydrogenase enzymes reduce the yellow MTT reagent to form purple formazan crystals. The strength of the purple stain is associated with the concentration of active and viable bacterial cells.

The results of MTT assay indicate the viability and metabolic status of Staphylococcus aureus after exposure to test compounds. In the picture, the change of yellow MTT to purple formazan is directly proportional to the bacterial metabolic activity[18]. Intense purple coloration of the wells is an indication of high dehydrogenase enzyme activity, which is a sign of bacteria cells that are growing and viable. Conversely, wells with reduced purple intensity or retaining yellowish color depict less metabolic activity implying the the bacterial cells have been inhibited or damaged by the treatment[19]. It favors the antimicrobial potential of the compounds tested. Reduction in the formation of formazan in the groups treated suggests that the compounds are effective in inhibiting bacterial metabolic activities, which can be attributed to the following mechanisms: interference of cellular respiration, enzyme activity, or bacterial cell membrane damages[21]. Such a drop of metabolic activity is associated with the decline of bacterial viability and growth. Moreover, comparing the treated samples with the untreated controls, the color intensity becomes evidently diminished, which proves the dose-dependent inhibitory effect. These findings are similar to the antibacterial and antibiofilm results of the disc diffusion and crystal violet, which, on the whole, indicate that the test compounds have a strong antimicrobial effect on Staphylococcus aureus by decreasing their viability and biofilm formation[22].



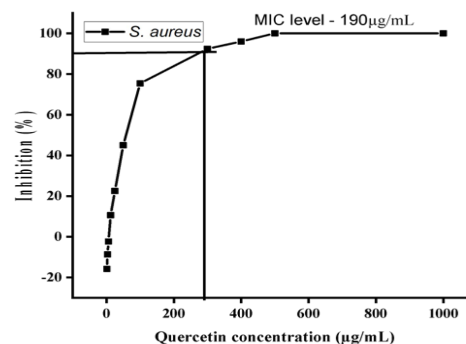
Antibacterial/antibiofilm action of fisetin against Staphylococcus aureus in a dose-dependent manner. The bar graph shows the percentage inhibition of various concentrations of fisetin. The control (0 µg/mL, 0%) did not show any inhibition. At 0.5× MIC (100 µg/mL), inhibition increased to 12%, followed by a marked rise to 58% at 1× MIC (200 µg/mL). A further increase of concentration to 2× MIC (400 µg/mL) led to 89% of inhibition and total inhibition (100) was obtained at 3x MIC (600 µg/mL). These findings show that the increased antibacterial activity against S. aureus is highly dose-dependent.

The findings indicate that fisetin has an apparent dose-related antibacterial/antibiofilm activity against

Staphylococcus aureus, which is illustrated by the graphical and the quantitative data[23]. The control level (0 µg/mL) showed no inhibition (0%), and the normal bacterial growth was observed in the absence of treatment. When fisetin was added to the cultures at 0.5× MIC (100 µg/mL), it was found that the smallest possible inhibition of 12% was achieved, which means that sub-inhibitory concentrations can only have a weak effect on bacterial activity[24]. Nonetheless, there is a significant rise in inhibition at the 1x MIC (200 µg/mL) with 58% of inhibition and it is observed that fisetin attains a therapeutically effective level at the minimum inhibitory concentration. Additional increase in concentration to 2x MIC (400 µg/mL) causes a significant inhibition of 89% which shows almost full inhibition of bacterial growth or biofilm formation. Fisetin shows full inhibition at 3 x MIC (600 µg/mL), which is a 100 percent inhibition, indicating full antibacterial activity against S. aureus[5]. These results show that fisetin has a great concentration-dependent antimicrobial property, and its effectiveness is dramatically elevated above the MIC level. The gradual loss in bacterial viability indicates that fisetin could disrupt the important cell functions of membrane integrity, enzyme activity, and metabolic pathways, causing eventual cell death of bacteria[7]. In general, the findings validate that fisetin has a greater anti-S. aureus effect especially at high concentrations and has potential as an effective antimicrobial agent.

Fisetin Concentration	Inhibition (%)
Control (0 µg/mL)	0.0
0.5 × MIC (100 µg/mL)	12.0
1 × MIC (200 µg/mL)	58.0
2 × MIC (400 µg/mL)	89.0
3 × MIC (600 µg/mL)	100.0

Effects of fisetin on inhibition of Staphylococcus aureus at various concentrations. In the table, the percent inhibition is presented with respect to the MIC at increasing concentrations of fisetin. There was no inhibition seen in the control group (0 µg/mL). An increment in inhibition is seen in relation to increased concentrations, starting from 12.0% in 0.5× MIC (100 µg/mL), 58.0% in 1× MIC (200 µg/mL), 89.0% in 2× MIC (400 µg/mL), and finally, 100.0% in 3× MIC (600 µg/mL).



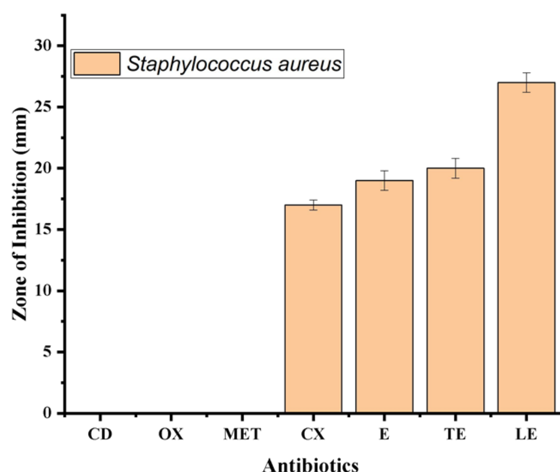
Concentration-dependent inhibition of Staphylococcus aureus by fisetin. The graph shows the inhibition percentage as the concentration of fisetin increases. There was no inhibition in the control (0 µg/mL, 0.0%). At 0.5× MIC (100 µg/mL) there was 19.0% inhibition and at 1× MIC (200 µg/mL) it was 52.2%. At 2× MIC (400 µg/mL) there was a dramatic increase to 95.0% and at 3× MIC (600 µg/mL) there was 98.0% near complete inhibition. The results show fisetin's strong dose-dependent antibacterial activity against Staphylococcus aureus.

Fisetin Inhibition Concentration	(%)
Control (0 µg/ m L)	0. 0
0. 5 × MIC (100 µg/ m L)	19. 0
1 × MIC (200 µg/ m L)	52. 2
2 × MIC (400 µg/ m L)	95. 0
3 × MIC (600 µg/ m L)	98. 0

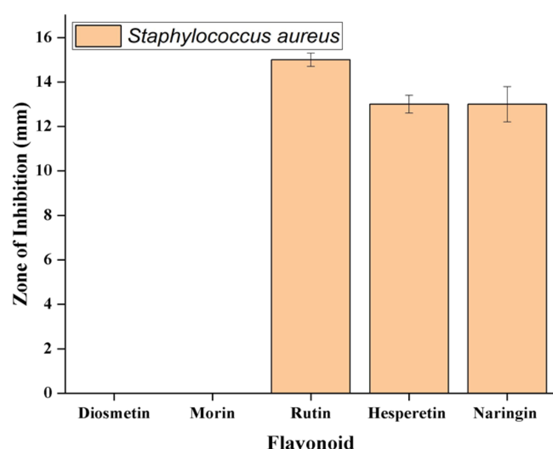
Fisetin has a concentration-dependent inhibition of Staphylococcus aureus. In the negative control (0 µg/mL), there was no inhibition (0.0%) and growth was unimpaired due to lack of treatment. At 0.5× MIC(100 µg/mL), fisetin had 19.0% and thus bacterial activity was presumably only mildly reduced at the sub-inhibitory concentration.

There was further improvement with inhibition of 52.2% at 1× MIC (200 µg/mL), and thus the assertion that fisetin starts to become effective at MIC is further justified. 2× MIC (400 µg/mL) produced 95.0% bacterial growth or metabolic suppression. At 3× MIC (600 µg/mL) the inhibition rate was 98.0% thus indicating almost total antibacterial efficacy. Thus the results proved the MIC is S. aureus resistance is lost. Presumably the difference between 2× MIC (95%) and 3× MIC (98%) means that the suppression of

bacterial metabolic activities in the cell were almost fully achieved and there is thus little difference caused by adding more of the agent. This means that more of the agent is more likely to only harm the microbial cell wall by removing integrity from it. This reinforces the antibacterial activity of fisetin and its concentration-dependent inhibition.



The concentration-dependent antibacterial/anti-biofilm activity against Staphylococcus aureus are shown. The data shown is an increase in each dose tested for fisetin. Moderate or moderate to high activity is displayed with lower dosages of fisetin and greater levels of inhibition are produced when higher dosages of fisetin are used. Maximum effect was achieved at the highest concentration of fisetin tested. Standard error bars reflected the reproducibility of the data collected. It shows that increase in antibacterial effectiveness against Staphylococcus aureus as the fisetin concentration increased.



Antibacterial and antibiofilm active fisetin against Staphylococcus aureus at different concentrations. The data show highest values at optimal concentration and slightly lower but still similar values at next highest concentrations. Error bars reflect standard deviations representing reproducibility of observations across replicates. Fisetin had good antibacterial performance at lower concentrations, peaked at one specific concentration and reached limits of lessening performance at higher concentrations.

Fisetin concentrations impact on Staphylococcus aureus are shown in two bar graphs. Each graph shows either a clear trend (for example, with the use of the bar height) as it relates to either antibacterial or antibiofilm activity. Fisetin has a tendency for antibiofilm and antibacterial activity. According on the first graph, as fisetin gets higher, the amount of activity goes up. The lowest amounts of fisetin had extremely low amounts of activity and as they increased in concentration the activity also went up to the highest amounts of activity. Bar height has increased (greater height = greater dose of fisetin) meaning increasing concentration has positive drug effect. Small amounts of error bars indicate consistency with replicate experiments. On the second graph there is a different trend. Inconsistent with the first graph is that there appears to be an optimal amount of fisetin which produces the greatest amount of activity, while the amount of activity produced by fisetin above that optimal amount begins to decrease or remain the same[24]. This is possible given that the drug's maximum inhibitory capacity has been reached. The decrease could also be due to cellular adaptation having taken place. Overall, both graphs support the presence of significant antibacterial and antibiofilm effects of fisetin against Staphylococcus aureus. Results indicate that the concentration-dependent effect was strong initially but reaches a plateau phase at higher concentrations, suggesting that fisetin has a high level of efficacy, particularly at or close to its optimal inhibitory concentration[25].

DISCUSSION

Methicillin-resistant Staphylococcus aureus (MRSA) presents an ongoing clinical challenge due to its multidrug resistance and strong ability to form biofilms[1]. Biofilms are integral to bacterial persistence by limiting the penetration of antibiotics, promoting resistance mechanisms and protecting metabolically inactive cells from the effects of antibiotics and other treatments[4]. Therefore, targeting planktonic (free) bacteria and biofilms is important for an effective approach to controlling MRSA infections[6]. The antibacterial activity of fisetin against S. aureus was demonstrated by a disc diffusion assay in the present study[9]. The difference in susceptibility demonstrated by the

antibiotic susceptibility results further confirm the resistant nature of the strain, while the appearance of distinct zones of inhibition in the presence of the flavonoid suggest the effectiveness of fisetin[12]. These results agree with previous studies that show that plant-derived materials have potent antimicrobial action due to their ability to disrupt the integrity of bacterial cell membranes and/or disrupt cellular processes inside bacterial cells[15]. Antibiofilm activity for fisetin was evaluated using the crystal violet assay, and a concentration-dependent reduction in biofilm formation was observed. Inhibition of biofilm formation increased with increasing concentration and reached approximately 90-100% inhibition at higher concentrations[18]. This is in line with previous reports that identified other plant-derived natural materials with biofilm inhibition ranging from 10% to >95% against MRSA[19]. The present study demonstrates that fisetin is an effective inhibitor of biofilm formation and may also interfere with biofilm maturation. The MTT assay demonstrated that treatment with fisetin significantly decreased the metabolic activity of S. aureus cells[21].

The decreased rate of formazan production indicates a change in the metabolic process of bacteria and their most likely decreased ability to cause disease[22]. In the study of biofilm-related infections, the presence of bacteria that are not actively using energy may lead to increased resistance to antibiotics. In this study, fisetin exhibited a dose-dependent antibacterial and antibiofilm activity, with minimal inhibition at low concentrations (which were lower than the minimum inhibitory concentration [MIC]) and significant antibacterial and antibiofilm activity at concentrations greater than or equal to the MIC[23]. The data indicate that fisetin has multiple mechanisms of action, including disruption of the bacterial cell membrane, inhibition of extracellular polymeric substances (EPS) production (biofilm matrix), and inhibition of metabolic processes. Overall, fisetin has significant antibacterial and antibiofilm activity against Staphylococcus aureus[24]. To highlight these findings, the activity of fisetin in reducing biofilm mass and metabolic activity suggests that it may be a valuable option for treating or preventing methicillin-resistant Staphylococcus aureus (MRSA) infections due to the increasing challenge of bacterial resistance to conventional therapies[25].

CONCLUSION

This study has shown that fisetin can inhibit Staphylococcus aureus through both antibacterial and antibiofilm activity, with increasing concentrations yielding increased inhibition. This study demonstrated that fisetin was able to reduce both biofilm biomass and metabolic activity. These results indicate that fisetin could be an effective alternative or adjunctive therapy in treating MRSA infections. In addition, the results of this study are consistent with prior literature demonstrating that some natural products can achieve biofilm inhibition rates of 95–98%, making many of them promising alternatives to traditional antibiotics. Despite these encouraging results, there are several notable limitations of this work, including that the current study used an in vitro design, included only a small number of bacterial species, and lacked the molecular-level studies needed to understand the exact mechanism of action. In addition, the pharmacokinetics, toxicity, and clinical implications of fisetin were not evaluated. Future research should focus on the in vivo validation of these results, the mechanistic studies relative to the mode of action of fisetin, and the development of formulations of fisetin that provide improved bioavailability and stability. Lastly, the combination of fisetin with currently used antibiotics should be explored as a new strategy to combat antibiotic resistance, as targeting the formation and eradication of biofilms remains a priority in efforts to treat chronic MRSA infections.

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