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Original article

Evaluation of the antibacterial and antibiofilm properties of quercetin against clinical isolates of methicillin-resistant *Staphyloccocus aureus*

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ABSTRACT

Background: Methicillin-resistant *Staphylococcus aureus* (*MRSA*) poses a serious clinical threat in hospitals. *MRSA* strains have acquired resistance to nearly every beta-lactam antibiotic because there has been a change in the penicillin-binding protein (PBP2a) encoded by the mec genes. **Aim of the work:** The objective of this study is to assess quercetin's antimicrobial and antibiofilm capabilities toward *MRSA* clinical isolates from Ain Shams University Hospitals. **Methods:** We obtained total of 40 *MRSA* isolates from the main microbiology lab of Ain Shams University Hospitals. Antimicrobial susceptibility testing and phenotypic identification were carried out, and the MIC of quercetin was determined against 40 *MRSA* isolates. Sub-MIC quercetin levels were used to determine its impact on biofilm formation. Biofilm formation was significantly reduced by quercetin compared to the control grown in the absence of quercetin. **Results:** *MRSA* clinical isolates are susceptible to the antimicrobial and antibiofilm effects of quercetin, and its inhibitory effects increase with its concentration. **Conclusion:** Quercetin shows antimicrobial and antibiofilm activities against *MRSA* clinical isolates.

Introduction

Staphylococcal cassette chromosome *mec* (SCCmec) is a genetic element that could be transmitted and contains mec genes as well as genes linked to antibiotic resistance [1]. The most prevalent variant is *MecA*, and previous study showed *MRSA* isolated in Ireland were found to include the divergent *mecA* homolog *mecC* [2].

The ability of *MRSA* to form biofilm, which has polymerizable mucopolysaccharide on its surface, is another significant cause of antibiotic resistance in *MRSA* and prevents antibiotics from entering bacterial cells. In addition, biofilm encloses the bacterial colonies near to each other increasing the horizontal transmission of genes responsible for resistance [3].

The goal of many drugs used to treat *MRSA* is to hinder biofilm formation. Hindering of biofilm synthesis is facilitated by various anti-biofilm agents with distinct structures, such as chelating agents, antibiotics, herbal compounds, and synthetic chemicals. This happens by too many mechanisms including blocking the quorum sensing mediated by N-acyl homo-serine lactones, inhibiting the stringent response exhibited by bacteria, eliminating the extracellular polysaccharide substance of biofilms, destroying peptidoglycan, causing biofilm dispersion, neutralizing lipopolysaccharides,

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altering membrane permeability, suppressing cell division, directly altering nucleic acid synthesis, and impeding the cyclic di-GMP signaling system [4].

In the pharmaceutical industry, plant secondary metabolites are commonly used as sources of preservatives, flavoring agents, medicinal compounds, coloring agents, and pesticides [5]. One of the major groups of secondary metabolites includes flavonoids and other phenolic compounds [6]. Natural substances extracted from plants have been found to be promising as prophylactic and therapeutic agents [7].

Flavonoids offer a variety of therapeutic uses, such as anti-inflammatory, anti-oxidant, anti-cancer, and anti-bacterial properties. In particular, anti-bacterial and anti-biofilm activities of various flavonoids were a major attention of many studies during the last decade [8].

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a flavonoid present in numerous plant foods; it can be found in onions, capsicums, cranberries, tomatoes, apples, and grapes [9]. Quercetin's antibacterial action mostly involves breaking down bacterial cell walls and modifying cell permeability, modifying protein production, inhibiting enzyme activity, and blocking RNA synthesis [10].

It is proven that quercetin has biofilm inhibitory effect on various pathogens by decreasing the total protein and viable cells in the biofilm [11].

The production of biofilms was influenced by the expression of adhesion-related genes *icaA* and *icaD*, whose expression was greatly reduced when quercetin ((10 μg/mL)) was added. Additionally, quercetin lowered the expression of the virulence-regulatory genes *sigB* and *sarA* as well as the quorum-sensing gene *agrA*. Communication between neighboring bacteria and biofilm production are subsequently reduced by qorum-sensing gene inhibition [12].

Thus, we will investigate quercetin's antimicrobial and antibiofilm properties against clinical isolates of *MRSA* in this study.

Material and methods

Type of the study

In vitro trial study.

Forty clinical isolates of *MRSA* were used in our study, which were obtained from the central microbiology laboratory at Ain Shams University Hospital. All *MRSA* isolates were stored at −80 ℃ in nutrient broth with 20% (vol/vol) glycerol.

We carried out our work from October 2022 to January 2023. Research Ethics Committee at Ain Shams University Faculty of Medicine approved the study (No. FMASU MS 520/2022). This study followed the principles of the Helsinki declaration.

Methods

Antimicrobial susceptibility testing

Using the disk diffusion method, antimicrobial susceptibility testing was conducted for the 40 *MRSA* clinical isolates in accordance with Clinical and Laboratory Standards Institute (CLSI, 2023) guidelines [13]. The clinical isolates were tested for their susceptibility to the following antibiotics (Oxoid, England): Ampicillin 10 μg, Amoxicillin/Clavulonic acid 20/10 μg, Meropenem 10 μg, Imepenem 10 μg , Cefepime 30 μg, Linezolid 30 μg , Levofloxacine 5 μg, Erythromycin 15 μg, Co-trimoxazole 25 μg , Doxycycline 30 μg and Clindamycin 2 μg. Methicillin resistance was confirmed using Cefoxitin (30μg) disk (Oxoid, England). *S. aureus* was known to be methicillin resistant when the zone of inhibition was ≤ 21mm, as illustrated in **(Figure 1)** [14].

Determination of the minimum inhibitory concentrations (MIC) of quercetin

The MIC of quercetin was determined using the broth micro-dilution technique in sterile microtiter plate [14,15]. Serial dilutions of quercetin were done in two folds manner using Mueller-Hinton broth (MHB) (Oxoid, UK) and a final volume of 0.1 ml of each concentration in each well was reached. We prepared a standard inoculum for each isolate using the direct colony suspension and diluted it with broth till it reached a concentration of 0.5 McFarland which was further diluted at a ratio of 1:20 to produce 10^6 CFU/mL. We introduced 0.01 mL of the prepared suspension into each well. Two types of negative controls were prepared, one of them was only broth which confirmed no contamination in our work, and the other type was quercetin in different concentrations with no organism added. The microtiter plate was incubated at 37°C for 24.

Using the resazurin microtiter plate assay, the minimum inhibitory concentration (MIC) of quercetin against *MRSA* clinical isolates was ascertained. Resazurin indicator in this assay (Sigma-Aldrich, Germany) changes in color from

blue to pink in the presence of viable cells. We introduced resazurin (0.015%) to all wells (30 µL per well) the plates were incubated for 2–4 h to observe color change, where the MIC was described as the dilution at which there was no color change. (blue resazurin color remained unchanged) **(Figure 2)**.

The sub-MIC concentration is the dilution that is below the minimum inhibitory concentration (MIC). It was used to test quercetin's ability to prevent biofilm growth.

Crystal violet microtiter plate assay for quantitative detection of biofilm formation

The organisms were inoculated in tryptic soy broth (TSB) containing 1% glucose using an overnight culture sample and incubated at 37°C for 24 hours. Each well of a sterile 96 well-flat bottom polystyrene tissue culture plate (Sigma-Aldrich Co. LLC, USA) was filled with 200 µl of the bacterial suspension along with control organisms (*Staphylococcus aureus* ATCC 25923 as a negative control and *Staphylococcus aureus* BAA1026 ATCC as a positive control). To ensure that media used are sterile and there is no non-specific binding, only broth was prepared as a negative control. After 24 hours, the plates were gently tapped to remove the contents of each well before being washed three times with 300 µl of sterile saline. The remaining adherent bacteria were exposed to hot air at 60 °C for 60 minutes for heat-fixation. After that, each well received 150 µl of crystal violet stain. After removing the excess stain and washing the plate, 150 µl of 95% ethanol was added to each well. After 30 minutes, the OD of staining adherent bacterial films was measured at 492 nm and 630 nm with a microtiter plate reader (**Thermo Fisher Scientific, America).** We conducted the test in triplicate manner and the average of results was calculated. After calculating OD values for all tested strains and negative controls and determining the cut-off value (ODc), the strains were divided into the following categories for interpretation: non biofilm producer (0): OD ≤ ODc, weak biofilm producer (1+): ODc <OD≤ 2×ODc, moderate biofilm producer (2+): 2×ODc <OD ≤ 4×ODc, strong biofilm producer (3+): 4×ODc <OD [13,15].

The phenotypic inhibitory activity of the sub-MICs of quercetin on biofilm formation

The effect of quercetin on biofilm formation was examined using the method mentioned previously but after treatment with sub-inhibitory concentrations of quercetin (MIC/2 and MIC/4) **(Figure 3)**.

$$\text{Inhibition rate} = 1 - \frac{\text{OD Treatment}}{\text{OD Control}} \times 100 \text{ [16].}$$

Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS version 25). The Kolmogorov-Smirnov test was used to assess normality for continuous variables. Descriptive analyses were performed to obtain the means, and deviations for quantitative data when it is normally distributed, and median, IQR for skewed data. Numbers and frequencies for qualitative data.

Different types of graphs were used according to the type and distribution of data (bar, pie and scatter plot), Bivariate analyses were performed using the Kruskal-Wallis test, and the chi-squared test for categorical variables. P value < 0.05 was considered significant.

Results

Forty *MRSA* isolates were obtained from central microbiology laboratory at Ain Shams University Hospital. As shown in **(Table 1)** the average age of patients from whom the *MRSA* isolates were collected was 39.1 ± 27.27, 62.5% were males and 37.5% females.

The *MRSA* isolates used in our study were obtained from various clinical samples. Most of isolates were collected from blood (15/40, 37.5%), followed by wound (11/40, 27.5%), pus (10/40, 25%), central line (3/40, 7.5%) and sputum (1/40, 2.5%).

According to antibiotic sensitivity patterns of the isolated strains, the highest resistance was reported to Ampicillin, Amoxicillin/Clavulonic acid, Ceftriaxone, Meropenem, Imepenem and Cefepime (100%), followed by Clindamycin (90%), Doxycycline (80%), both Erythromycin and Co-trimoxazole each (77%), Levofloxacin (65%), linezolid (53%). The antibiotic resistance pattern is displayed in **(Table 2)**.

All of the Forty isolates of *MRSA* were identified to be biofilm producers using the microtiter plate assay. About 62.5% (25/40) of the isolates were moderate biofilm producers (+2), 28% (11/40) were weak biofilm producers (+1) and 10% (4/40) were strong biofilm producers (+3) as shown in **(Figure 4)**.

For *MRSA*, quercetin's MIC values varied from 125 to 1000 µg/ml. Furthermore, even at sub-inhibitory doses, quercetin was able to

suppress *MRSA* biofilm formation, reducing MRSA antibiofilm production at MIC, MIC/2, and MIC/4 in comparison to the negative control. According to **(Table 3,4)**, there was a correlation between the antibiofilm effect of quercetin and its MIC, which was 59.59% ± 21.8% at 1/2 MIC and 48.41% ± 24.35% at 1/4 MIC.

Table 1. Socio demographic data (N=40)**.**

Age in years			
Mean± SD		39.1±27.27	
Median (IQR)		46.5 (11-62.5)	
Min-Max		0.01-83	
		N	%
Gender	Male	25	62.5%
	Female	15	37.5%
Specimen type	Pus	10	25.0%
	Blood	15	37.5%
	Central line	3	7.5%
	Wound	11	27.5%
	Sputum	1	2.5%

Table 2. Susceptibility testing results of *MRSA* to different antibiotics by the disc diffusion method.

Antibiotic class	**Antibiotic agent**	**Sensitive**	**Intermediate**	**Resistant**
Fluoroquinolone	Levofloxacin	14 (35 %)	0	26(65%)
Tetracyclines	Doxycycline	8(20%)	0	32(80%)
Macrolides	Erythromycin	9(23%)	0	31(77%)
Lincomycins	Clindamycin	4(10)	0	36(90%)
Oxazolidinones	Linezolid	19(47%)	0	21(53%)
Sulfonamides	Co-trimoxazole	9(23%)	0	31(77%)
Beta-lactams	Ampicillin	0	0	100
Beta-lactams	Penicillin G	0	0	100
Beta-lactams	Amoxicillin/ Clavulonic acid	0	0%	100
Beta-lactams	Ceftriaxon	0	0%	100
Beta-lactams	Meropenem	0	0%	100
Beta-lactams	Imepenem	0	0%	100
Beta lactams	Cefepime	0	0%	100

Table 3. Antibacterial and antibiofilm activities of quercetin against *MRSA* isolates**.**

Isolate number	Biofilm formation					
	MIC of QUER (µg/ml)	Control	Quercetin at 1/2 MIC	Rate of inhibtion	Quercetin at 1/4 MIC	Rate of inhibtion
1	1000	(2+)1.4	0.35	78%	0.51	64%
2	1000	(2+)1.75	0.36	81%	0.39	78%
3	1000	(2+)1.88	0.327	83%	0.42	78%
4	1000	(2+)1.247	0.282	96.60%	0.36	72%
5	500	(1+)0.943	0.162	78%	0.296	69%
6	1000	(2+)1.12	0.319	80%	0.32	80%
7	1000	(2+)0.51	0.36	28%	0.5	1%
8	1000	(2+)0.40	0.33	25%	0.3	25%
9	1000	(+2)0.31	0.27	10%	0.3	1%
10	1000	(+2)0.5	0.31	40%	0.3	40%
11	1000	(2+)0.6	0.21	66%	0.2	66%
12	1000	(2+)2	0.4	80%	0.4	80%
13	1000	(3+)2.1	0.3	85%	0.5	76%
14	500	(2+)1.1	0.4	63%	0.7	36%
15	1000	(2+)1.1	0.5	54%	0.6	45%
16	125	(2+)1.077	0.4	62%	0.6	44%
17	125	(1+)0.8	0.4	62%	0.39	51%
18	125	(1+)1.04	0.4	61%	0.67	35%
19	1000	(2+)2.1	0.6	71%	0.69	67%
20	1000	(3+)2	0.4	80%	0.5	75%
21	1000	(2+)1.1	0.5	54%	0.5	63%
22	1000	(2+)1.04	0.67	35%	0.7	32%
23	1000	(1+)0.5	0.2	60%	0.3	40%
24	1000	(1+)0.8	0.4	50%	0.4	50%
25	1000	(1+)0.4	0.3	25%	0.35	12.50%
26	1000	(1+)1.07	0.6	44%	0.8	26%
27	500	(2+)2.2	0.4	81%	0.4	81%
28	1000	(1+)0.32	0.2	38%	0.3	2%
29	1000	(2+)1.04	0.6	44%	0.7	32%
30	250	(2+)1.077	0.4	62%	0.4	62%
31	1000	(2+)2	0.4	80%	0.7	65%
32	500	(2+)0.8	0.3	62%	0.6	25%
33	1000	(1+)0.6	0.4	34%	0.4	34%
34	500	(2+)22	0.4	82%	0.7	69%
35	250	(2+)2	0.4	80%	0.5	75%
36	1000	(1+)0.51	0.33	35%	0.42	18%
37	1000	(3+)2	0.3	85%	0.6	70%
38	250	(2+)1.04	0.6	44%	0.6	44%
39	500	(3+)2.2	0.5	80%	0.65	28%
40	125	(2+)0.4	0.3	25%	0.3	25%

Table 4. Quercetin antibiofilm effect and % of inhibition.

Quercetin antibiofilm effect 1/2MIC% of inhibition	**Mean + SD**	59.59%±21.81%
	Median (IQR)	62.00% (425-80%)
	Min- Max	10%- 96.50%
% Quercetin antibiofilm effect 1/4MIC%of inhibition	**Mean + SD**	48.41%±24.35%
	Median (IQR)	47.50% (30% - 69.5%)

Figure 1. Muller Hinton agar showing methicillin-resistant *Staphylococcus aureus* using cefoxitin disc.

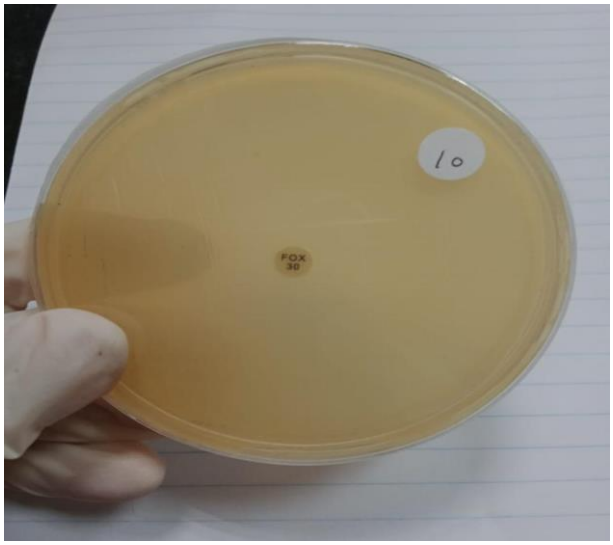

Figure 2. Determination of MIC for quercetin against *MRSA* clinical isolates. After the period of incubation, resazurin dye was added. Column 12 confirms no contamination occurred while preparing the plate. Column 11, a negative control shows a change of resazurin natural color (blue/purple) to the reduced form (red-colorless). The highest concentration incorporated into the plate is 1000 ug/ml and the lowest achieved through double serial dilution is 62.5 ug/ml-1. Column 1 shows no color changes therefore concentration of quercetin in that column was taken as the MIC value.

Figure 3. Quantitative crystal violet microtiter plate assay showed the effect of sub-MIC of quercetin on the biofilm formation of *MRSA* isolates. Columns 1,2,3,7,8,9 showed that the isolates were more strongly stained, indicating stronger ability to form biofilm mass. While isolates in columns 4,5,6,10,11,12 showed decrease in the intensity of staining, indicating inhibition of biofilm formation by quercetin. Row (H) columns 7,8,9,10,11,12 was negative control.

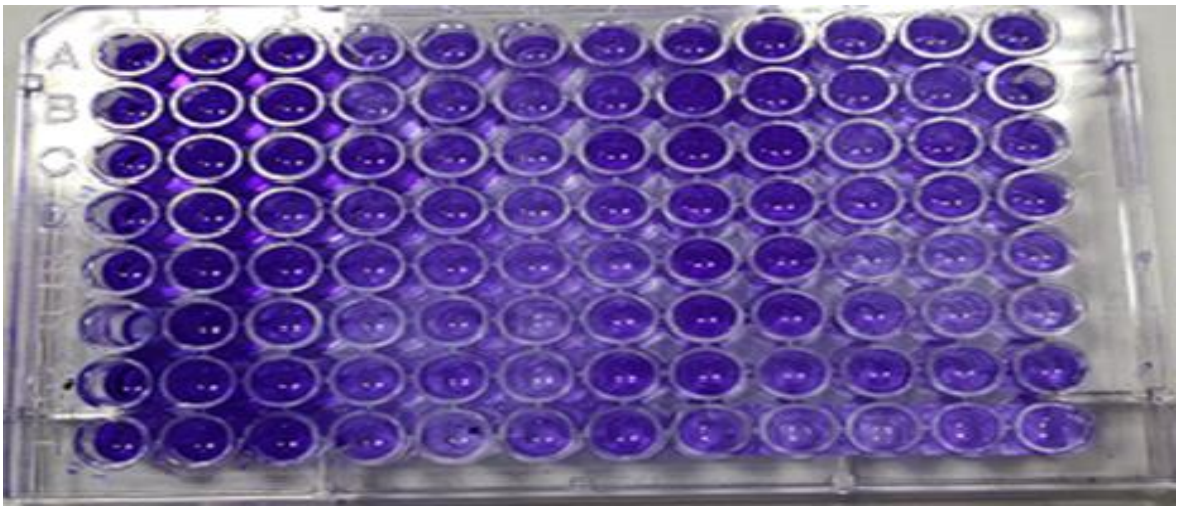

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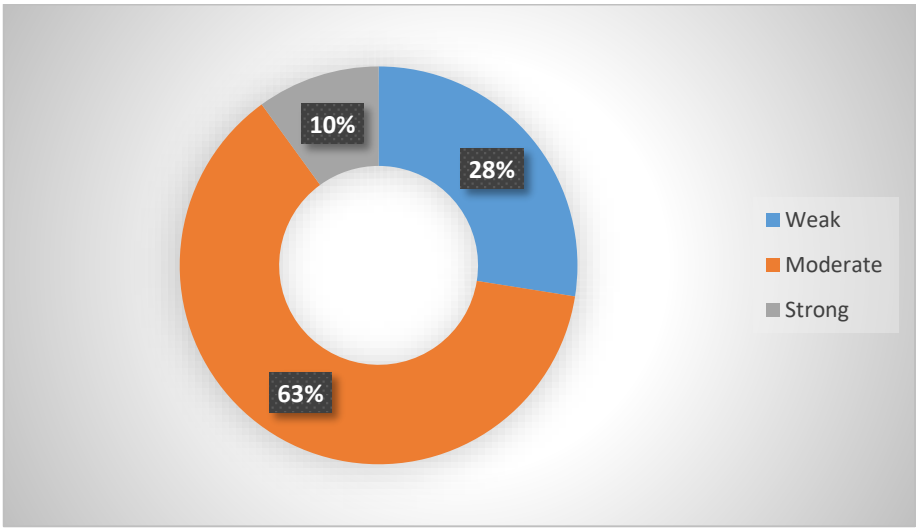
Figure 4. Distribution of biofilm formation among *MRSA* clinical isolates.



Discussion

Methicillin-resistant *S. aureus* (MRSA) is a multidrug-resistant variant of *S. aureus* that has been linked to more severe lung dysfunction, prompting recommendations for its elimination. Antimicrobial therapy is complicated by bacteria's adaptive abilities and diverse features, such as the production of biofilms [17].

One significant phytochemical that is a member of the flavonoid polyphenol group is quercetin. Although it can be found in a variety of fruits, vegetables, drinks, flowers, leaves, seeds, and other materials, the onion (Allium cepa) is the most abundant source. Apples, red wine, kales, tea, and tea are other sources of quercetin [18].

Although quercetin may have antibacterial properties against various bacteria, fungi, and viruses, there is limited research on its effectiveness against *MRSA* biofilms in clinical isolates from Egypt [19].

Our objective was to investigate the antimicrobial and antibiofilm effects of quercetin on 40 clinical isolates of *MRSA*.

The mean age of the patients from whom the *MRSA* isolates were obtained was 39.1±27.27,

with 62.5% of them being males and 37.5% being females, based on our sociodemographic data of the 40 clinical samples.

Mazhar et al. [20] investigated *S. aureus* isolated from clinical specimens in Jordan, finding that 57% of it was found in males while only 43% was found in females. The percentage of *MRSA* strains was the same for both genders, but *S. aureus* was more prevalent in males than females. In our investigation, the majority of the isolates (37.5%) came from blood. However, a higher percentage of *MRSA* (40%) was found in ear swabs according to **Hafeez et al.** [21].

Isolates used in our study were collected from different clinical samples obtained from different sites, most of them were found in blood (37.5%) followed by wound (27.5%), pus (25%), central line (7.5%) and sputum (2.5%).

A study done by **Vysakh and Jeya** [22] found that the lowest percentage of *MRSA* isolates were from blood specimens. However, the highest percentage were obtained from wound exudates (91%), followed by blood (5%), urine (2%), and respiratory (2%) specimens.

Numerous factors, including the patient population, the kinds of skin normal flora, the methods used to collect the specimens, and the quantity of specimens, can be blamed for the variations in clinical specimens.

Most pathogenic *MRSA* strains are multidrug resistant, this along with the biofilm formation ability of *MRSA* strains, potentiates the overall resistance to antibiotics, resulting in treatment failure. In our study, all isolates were tested for antimicrobial susceptibility and the highest resistance was reported to Ampicillin, Amoxicillin/Clavulonic acid, Ceftriaxone, Meropenem, Imepenem and Cefepime (100%), followed by Clindamycin (90%), Doxycycline (80%), both Erythromycin and Co-trimoxazole each (77%), Levofloxacin (65%) and linezolid (53%).

Other studies reported varying susceptibility patterns of *MRSA*, with some studies reporting similar resistance to oxacillin, penicillin, and erythromycin which is similar to our result, and yet *MRSA* isolates in these studies were 100% susceptible to linezolid [23].

Quercetin exhibits different minimum inhibitory concentrations (MIC) against *MRSA* which proves the antimicrobial activity of quercetin against *MRSA*.

In our study, the antibacterial effect of quercetin at MIC 1000 µg/ml, was 67.50%(27/40). Through further dilution, the antibacterial effect of quercetin *MRSA* starts to decrease; where at 500 µg/ml quercetin the antibacterial effect was 15%(6/40), at 250 µg/ml was 7.5%(3/40) and at 125 µg/ml was 10%(4/40).

A study done by **Da Costa Júnior et al.**[23] showed that MIC of quercetin was 500 µg/ml against *MRSA*, which supports our findings.

Similar to our study, **Rauha et al.**[24] observed that quercetin exhibited antimicrobial activity at concentration of 500 µg/ml against ATCC strains of many different species: particularly, *S. aureus*, determined by the disc diffusion method.

On the contrary, **Nitiema et al.** [25] examined quercetin's antibacterial activity using the agar diffusion method at a 1000 µg concentration and found no activity against bacterial strains that cause gastroenteritis. This could be because the researchers tested the substance on different strains of bacteria.

In the present study, the biofilm formation was detected in all the 40 clinical isolates, about 62% (25/40) of the isolates were moderate biofilm producers, 28% (11/40) were weak biofilm producers, and 10% (4/40) were strong biofilm producers. This is consistent with a similar study conducted by **Da Costa Júnior and his colleagues** [23] who observed that, all clinical isolates in their study were biofilm-producers, 4.5% were classified as weak biofilm producers, 45.5% were strong biofilm-producer and 50% were moderate biofilm-producers.

However, previous studies showed that some *MRSA* isolates were non biofilm producers. A study performed by **Silva et al.** [26] revealed that only 80.5%, 77.6% and 58.3%, of *MRSA* isolates from bacteremia, diabetic foot infections and osteomyelitis patients respectively were biofilm producers. **Leshem et al.** [27] found that 40.5% of *MRSA* isolates were non biofilm producers and the remaining 59.5% were either strong or weak producers, however, about half of the *MRSA* isolates in this study were collected from screening nasal swaps and the other half were clinical isolates from patients with *MRSA* infection, which might explain the high percentage of non-biofilm producing strains in this study.

Several studies showed that quercetin has strong antibiofilm effects, one of these studies by

Yang et al. [28] reported that quercetin strongly inhibits the formation of biofilm of *S. aureus* ATCC 6538 strain at concentration as low as 50 µg/ml.

Another study conducted by **da Costa Júnior and his colleagues** [23] regarding the quercetin antibiofilm activity, they reported that quercetin reduces the biofilm production by *S. aureus* at MIC, MIC/2 and MIC/4, when compared to the negative control ($p < 0.05$), where they found that, quercetin, at MIC, reduced about 53.2%, 59.7 % of *MRSA* and *VRSA* biofilm production. At MIC/2, quercetin reduced 48.67 %, 45.7 % of the bacterial biofilm of *MRSA*, *VRSA*. At MIC/4, quercetin reduced 42.2% , 40.2% the bacterial biofilm of *MRSA*, *VRSA*.

Our study showed that the rates of biofilm inhibition by quercetin were 59.59%% at 1/2 MIC and 48.41% at 1/ 4 MIC. The previous findings showed that the antibiofilm effect of ½ MIC was more than that in case of using ¼ MIC of quercetin.

According to ***da Costa Júnior and his colleagues***[23], the rate of inhibition of *MRSA* biofilm formation by quercetin at ½ MIC was 48.67%. While when they used the sub inhibitory concentration of MIC/4, quercetin reduced 42.2% of the bacterial biofilm of *MRSA*, which supports our study results.

Conclusion

The importance of our results in the evaluation of the antibiofilm activity of quercetin was to prove that this molecule, is able to inhibit the formation of biofilm even in sub-inhibitory concentrations. So, we can affirm that quercetin exhibited a promising antibacterial and antibiofilm activity against *MRSA*. Ultimately, more research is needed to evaluate quercetin's antibacterial effectiveness in infections brought on by *Staphylococcus spp*. in vivo.

Conflict of interest

There is no conflict of interest stated by the authors.

Authors' contribution

All of the listed authors participated in the work and approved it for publication.

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