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Antimicrobial Efficacy of Quercetin against Biofilm Production by *Staphylococcus Aureus*

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ABSTRACT: ***Background:*** *The bad administration of antibiotics represents a worldwide health issue as it leads to the emergence of multidrug-resistant microbes including Staphylococcus aureus, which represents a significant challenge for public health since it increases its ability to cause potentially fatal infections.* ***Objective:*** *This study aims to reduce the bacterium's ability to form biofilms and antibiotic resistance.* ***Methods:*** *Thirty Staphylococcus aureus isolates were isolated from different clinical samples in Baghdad city and their ability to form biofilm was tested using the Microtiter Plate method. Out of 30 isolates, eight strong biofilm producer isolates were further investigated for their antimicrobial activity and biofilm formation with the impact of quercetin.* ***Results:*** *The results showed the ability of quercetin to inhibit bacteria and had an antibiofilm activity that was determined through investigation of the minimal inhibitory concentration by taking different concentrations. The anti-biofilm activity of quercetin was determined for the eight isolates and the results showed that biofilm formation was reduced by 100% using quercetin at a concentration of 3.1 mg/ml.* ***Conclusions:*** *Quercetin represents a suitable alternative to synthetic antimicrobials with the increased rate of drug-resistant bacteria among clinical samples with highly effective ability as anti-biofilm formation among strong biofilm producers of S. aureus..*

KEYWORDS: *Staphylococcus aureus; Multidrug-resistant microbes; Biofilm; Quercetin; Anti-biofilm activity*

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is related to gram-positive bacteria, which pose a serious threat to world health [1]. Around (25-30%) of healthy individuals were discovered to colonize with *S. aureus* and worldwide concern emerged for treating contagion caused by these bacteria [2]–[4]. The *S. aureus* has a broad spectrum of virulence factors [5], which are accountable for their pathogenicity including surface proteins, biofilm, exoenzymes, exotoxins, and exfoliative toxins [6], [7]. These bacteria are specially located in the skin and mucous membranes and can develop into opportunistic pathogens that are more virulent and lead to skin diseases or bacteremia [8]. The bacteria's capacity to attach to different tissue types by forming the biofilm protects them from applied therapies and serves as a protective way to defend and adapt to their environment [9]. The biofilm is a matrix of extracellular polymer that surrounds microbial cell populations enhances their adhesion to the surfaces and represents an ideal barrier against the antibiotics and assists the bacteria to avoid the immune system [10], [11]. The bacteria that produce the biofilm can defend the host mechanisms during their growth as well as protect themselves from opsonophagocytosis. This leads to tolerance to all traditional antimicrobials that predominately eradicate free-floating, single-cell (planktonic) bacteria especially those related to interaction with the biological materials of the cell wall making them a major worry in nosocomial infections, and became a public health risk [12].

Quercetin is an essential phytochemical compound that belongs to the flavonoid group (polyphenols), which is widely found in different fruits, vegetables, and beverages. The good plentiful source of quercetin is onion (*Allium cepa*), as well as tea, wine, kale, and apples are further sources of quercetin [13]. The unlimited use of antibiotics against bacterial infection led to the emergence of multidrug

resistance (MDR) bacteria which has become a hard challenge to the pharmaceutical industry [14]. In recent studies, the bioactive compounds that have been isolated from different sources proved to have a significant effect on human health as well as it could be an alternative strategy for inhibiting microorganisms including fungi and viruses [15]. Quercetin has antimicrobial action against a broad spectrum of bacterial strains, especially those involved in gastrointestinal, urinary, and integumentary systems [16]. The studies showed that quercetin can affect Gram-positive bacteria more than Gram-negative, this may relate to the differences in quercetin susceptibility and partially attributed to the composition differences of cell membranes between the Gram-positive and negative bacteria. On the other hand, some quercetin byproducts provide strong antibacterial potency against Gram-negative bacteria more than the other type [17].

MATERIALS AND METHODS

Samples Collection

A total of 135 (skin, nasal, and wound) samples were collected from patients and healthcare workers of different ages and genders. These samples were collected from November 2021 to February 2022 from Al-Numan Teaching Hospital and Central Child Teaching Hospital.

Isolation and Identification of *Staphylococcus aureus*

The isolates were identified according to the standard laboratory tests and Bacterial isolates identification was carried out by the VITEK-2 system.

Antibiotic Sensitivity Test for *Staphylococcus aureus* Isolates

The antibiotic susceptibility test was performed by the Kirby-Bauer disk-diffusion method for 10 antibiotics (Erythromycin, Cefoxitin, Ciprofloxacin, Penicillin, Ceftazidime, Gentamicin, Clindamycin, Azithromycin, Rifampin, and Tetracycline) as following: a bacterial suspension was prepared equivalent to 0.5 McFarland turbidity (1.5×10^8 CFU/ml). A cotton swab was submerged in the prepared suspension and subcultured on Muller Hinton agar plates. The antibiotic discs were placed in agar and pressed to ensure contact with the agar. Subsequently, the plates were incubated for 24 hours at 37 °C and the inhibition zone was measured. The results were interpreted according to the Clinical Laboratory Standards Institute (CLSI, 2021).

Effect of Quercetin on Bacterial Growth

Quercetin as an antibacterial agent was applied as follows by using the Agar-well diffusion method [18] and Quercetin was obtained from Sigma (USA). First, Muller-Hinton agar plates were inoculated with indicator isolates (*S. aureus*) by using some colonies from overnight culture and added to 5ml of normal saline to adjust the bacterial suspension to 0.5 McFarland turbidity equivalent to 1.5x108 CFU/ml. After that, the bacteria were spread by sterile cotton swab on all Muller-Hinton agar surfaces, the swab was streaked across the medium surfaces. Then using a sterile cork borer, five wells of about 6 mm diameter were aseptically cut on the agar plate. A volume of 50 mg/ml of quercetin was added to the wells. Finally, the plates were incubated overnight at 37 °C and the diameter of zones of inhibition was measured by millimeters.

Determine of Minimal Inhibitory Concentration of Quercetin

The micropipette was used to dispense 100 µl of the medium into each well of a microtiter plate except the first well added 200 µl. Following that, 100 µl of 2x extract solutions was pipetted into all wells in column 1 (far left of the plate). Then, 100 µl from column 1 was added to column 2. After that, 100 µl of column 2 was transferred to column 3 and was repeated until column 10. 15 µl of bacterial growth was dispensed into all wells except column 12 (contraceptive control and blank for the plate scanner). Then, plates were incubated at 37 °C or other desired temperature. When appropriate growth is obtained (24 hours) examine all the plates with GloMax® Discover Microplate Reader on 600 nm. MIC can be used as the lowest probable concentration of the drug [13].

Detection of Antimicrobial Efficacy of Quercetin against Biofilm Production by Microtiter Plate Assay

The *S. aureus*' ability to form biofilm was detected by microtiter plate assay using a method called crystal violet staining method. In brief, all 96-well flat-bottomed sterile polystyrene microplate wells which had 180 µL of Mueller–Hinton broth included with 1% glucose were administered with 20 µl from suspended bacteria of 0.5-0.7 McFarland (1.5×10^8 CFU/ml). The plates were incubated for 24 h at 37 °C. After that, the liquid media was removed, and the stuck cells underwent two cycles of washing by phosphate-buffered saline (PBS) then the wells were dried for 1hr or less at 60 °C. Following that it was coated for 15 min with 150 µL of 2% crystal violet. As a result, the wells were cleaned of crystal violet stain by rinsing them twice with PBS. After the air-drying process of the plate, the dye of biofilms that stuck in the walls of the microplate was re-solubilized by 150 µL of 95% ethanol. Finally, the read was recorded at 570 nm by spectrophotometer [19]. The results are interpreted as the following:
Strong biofilm producer ($4 \times$ ODc $<$ OD), Medium biofilm producer ($2 \times$ ODc $<$ OD $\leq 4 \times$ ODc), Weak biofilm producer (ODc $<$ OD $\leq 2 \times$ ODc), non-biofilm producer (OD $\leq$ ODc).
The optical density cut-off value (ODc) was determined to be three standard deviations (SD) above the mean of the optical density (OD) of the negative control as shown in the following formula:
$ODc =$ average OD of negative control $+ (3 \times SD$ of negative control).

RESULTS AND DISCUSSION

Samples collection

The clinical samples were obtained and collected from different sources, one hundred and thirty-five from Baghdad hospitals. Thirty isolates (23%) were identified as *S. aureus* by the traditional culture methods, biochemical and microscopic examination as well as the VITEK® 2 Compact system.

Antibiotic Sensitivity Test for *Staphylococcus Aureus* Isolates

The *S. aureus* isolates in this study showed different degrees of resistance against the antibiotics used. Ceftazidime and tetracycline antibiotics were the least active as the resistance rate was 76.7% and 66.7%, respectively. However, the highest sensitivity rate for *S. aureus* isolates was against ciprofloxacin and penicillin (86.7%). Furthermore, gentamicin and rifampin sensitivity rates were 76.7% for both. The sensitivity rate of *S. aureus* against azithromycin, clindamycin, erythromycin, and cefoxitin is 73.3%, 66.7%, 56.6%, and 50%, respectively. Nine isolates were recorded as multidrug-resistant (MDR) in this study.

Antibacterial Activity of Quercetin on *Staphylococcus Aureus*

Depending on the inhibition zone formed by the activity of (50 mg/ml) of quercetin against *S. aureus* isolates, the results showed that the quercetin had an effective antibacterial activity with an inhibition zone of (11 mm) as shown in Figure 1 this result was closely related to [17] who recorded (50 mg/ml) as the inhibition concentration of quercetin *S. aureus* isolates.

Quercetin has an antibacterial against many bacterial strains, the antibacterial ability of Quercetin has been associated with its solubility as it can interplay with the bacterial cell membrane. This can be determined by the presence of quercetin's hydroxyl groups [20]. The studies showed that quercetin could be more effective on Gram-positive bacteria than Gram-negative and this might be associated with the difference in cell membrane structure between them [21]. In general, the sulfuration and phosphorylation of quercetin at various hydroxyl groups may be capable of reducing or enhancing its solubility, thus changing its antibacterial activity to certain types of bacteria.

A recent study by [21] determined that Quercetin has an observed effect on disarranging the structure cell wall and cell membrane of *S. aureus* and *E.coli*, the treated *S. aureus* showed numerous structural abnormalities in the damaged cell wall such as cell distortion, thinning of the cell membrane, less of endochylema contents, chromatin lysis and irregular endochylema density, also notice a nuclear cavitation.

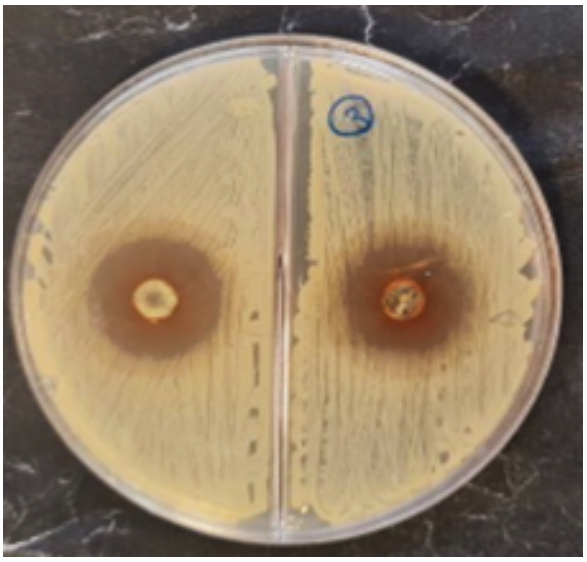

Figure 1. Antibacterial activity of quercetin against *staphylococcus aureus* with 50 mg/ml and incubation at 37 °C for 24 hrs.

Determination of Minimal Inhibition Concentration (MIC) of Quercetin against *S. Aureus* Isolates

Depending on the results obtained for the antibacterial activity of the quercetin at a concentration of 50 mg/ml could suppress the growth of *S. aureus* isolates. As a result, we measured the MIC by taking a concentration of Quercetin from (50, 25, 12.5, 6.3, 3.2, 1.56, 0.78, 0.39, 0.20, 0.10 mg/ml) on nutrient broth culture media. Table 1 summarizes these results, Quercetin gave minimum inhibition concentration on *S. aureus* isolates at a concentration of 3.1 mg/ml, The result was closely similar to the [13] which found that the MIC for *S. aureus.* while *P. vulgaris* and *E. coli* could not grow at concentrations of 30 mg/ml and 40 mg/ml.

Table 1. Measuring the minimum inhibition concentration of Quercetin against *S. aureus* isolates

Conc. mg/ml	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6	Isolate 7	Isolate 8
50.0	0.00	-0.04	-0.06	-0.01	0.00	-0.04	0.01	-0.09
25.0	-0.16	-0.02	0.06	0.02	0.06	0.00	0.06	-0.05
12.5	-0.19	0.06	-0.08	-0.01	0.05	0.03	0.09	-0.07
6.3	-0.05	0.08	-0.12	0.02	0.01	0.01	0.11	-0.08
3.1*	-0.15	0.09	0.08	0.10	0.10	0.09	0.10	0.09
1.56	0.86	0.87	0.86	0.67	0.72	0.69	0.66	0.71
0.78	0.86	0.90	0.88	0.78	0.75	0.75	0.71	0.75
0.39	0.92	0.93	0.91	0.82	0.80	0.82	0.75	0.78
0.20	1.03	1.04	1.06	0.87	0.85	0.88	0.79	0.87
0.10	1.47	1.15	1.34	1.01	1.10	0.98	0.90	0.92
Control	1.80	1.30	1.70	1.16	1.45	1.19	1.12	1.54

* The minimum inhibition concentration of the quercetin against *S. aureus*

Inhibition of the Biofilm Formation by Quercetin

Figure 2 shows the biofilm formation in the microtiter plate by *S. aureus* isolates, the microtiter plate method is the most frequent method used for the detection of biofilm formation [22]. The quercetin (3.1 mg/ml) inhibits the biofilm formation of eight isolates at a 100% inhibition rate as shown in Table 2 for data analysis by microplate reader after measured by spectrophotometer at 570 nm.

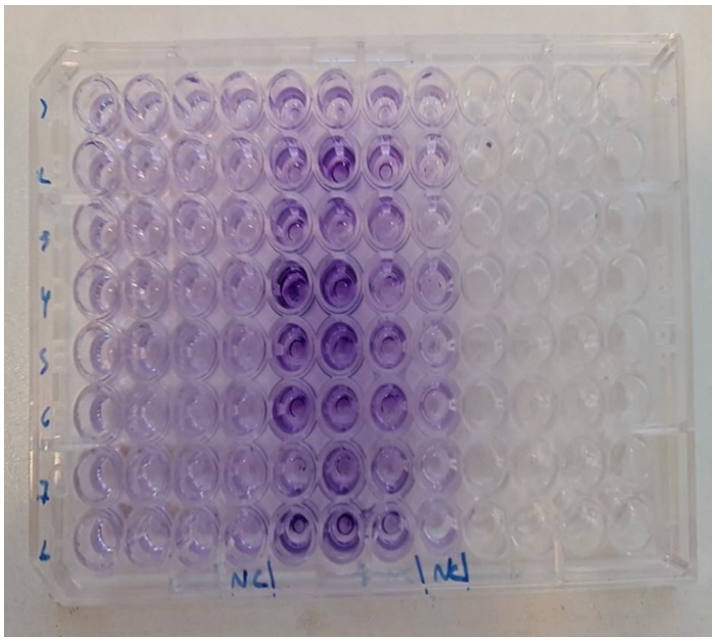

Figure 2. The biofilm formation in the microtiter plate method by *S. aureus* isolates

Table 2. Data analysis for the inhibition of biofilm by quercetin

Isolate	OD1	OD2	OD3	Average	OD isolate	ODC	2×ODc	4×ODc
1	0.14	0.14	0.12	0.13	-0.07	0.20	0.40	0.81
2	0.12	0.11	0.12	0.12	-0.08	0.20	0.40	0.81
3	0.13	0.12	0.12	0.12	-0.08	0.20	0.40	0.81
4	0.12	0.11	0.12	0.12	-0.08	0.20	0.40	0.81
5	0.11	0.12	0.12	0.12	-0.08	0.20	0.40	0.81
6	0.13	0.11	0.12	0.12	-0.08	0.20	0.40	0.81
7	0.13	0.12	0.12	0.13	-0.08	0.20	0.40	0.81
8	0.12	0.12	0.14	0.13	-0.07	0.20	0.40	0.81
Negative Control	0.13	0.13	0.13	0.13	-0.07	0.20	0.40	0.81

Quercetin showed perfect anti-biofilm activity toward the strong biofilm producers of *S. aureus* isolates as shown in Table 2, it agreed with the result of [13] who reported that Quercetin prevented the formation of biofilm in drug-resistant *S. aureus* as well as suppression the outcome of genes that responsible for bacterial adhesion. Another study [20] showed that Quercetin can reduce the formation of biofilm and has an effect on the expression of virulence genes in multidrug-resistant *S. aureus*. Quercetin can prevent biofilm formation by reacting with the biological pathways in bacteria, especially the quorum sensing, subsequently, it can prevent bacterial adhesion to target organs [23]. Recent studies have shown that Quercetin extract could eliminate the activities of several virulence enzymes, subsequently, it can prevent bacterial virulence [24]. Additionally, Quercetin can inhibit the action of the coagulase enzyme which is an essential factor of virulence in *S. aureus*. Additionally, Quercetin can be a protection for rats from catheter-associated *S. aureus* infections [25].

CONCLUSION

Quercetin represents a suitable alternative to synthetic antimicrobials with an increased rate of drug-resistant bacteria among clinical samples. This study showed that quercetin is a highly effective ability as an anti-biofilm formation among strong biofilm producers of *S. aureus*. The obtained results

demonstrated that quercetin could suppress the growth of *S. aureus*, as it can be an alternative way to inhibit the formation of biofilm in *S. aureus*.

SUPPLEMENTARY MATERIAL

None.

AUTHOR CONTRIBUTIONS

Rayhana S. Najim: Conceptualization, methodology, editing, and software. Mohsen Hashem Risan: Formal analysis, resources, and investigation. Dhafar Najim Al-Ugaili: Validation, formal analysis, writing review.

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None.

DATA AVAILABILITY STATEMENT

Data is available in the article.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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