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Anti-biofilm activity of Coumarin compound and evaluate effect on the gene expression of *icaA* and *cifA* genes in *Staphylococcus aureus*

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Abstract : The aim of this work is to examine the anti-biofilm capabilities of coumarin compound and apply them to specific virulence genes that contribute to the formation of biofilm in multidrug-resistant *Staphylococcus aureus*. The lab. of the UOB Institute of Genetic Eng. and Biotech.provided Twenty-five isolates of S. aureus. Ten isolates of *S. aureus* that were resistant to several drugs were chosen, and their capacity to produce biofilm was examined using the micro-titer plate method. The lab. of the UOB Institute of Genetic Eng. and Biotech. provided these isolates. By growing the isolates on Mannitol salt agar and applying the VITEK-2 technology, the diagnosis was verified. According to the findings, every isolate had a high capacity for biofilm production. Furthermore, a number of tests were carried out on the coumarin compound, such as determining the total phenolic content, assessing the antioxidant activity, evaluating the biofilm formation activity, and comparing isolates treated with a coumarin substance with isolates that were not in order to analyse the expression of the *icaA* and *cifA* genes. The findings demonstrated a progressive rise in the Coumarin compound's total phenolic content as concentration rose, with significant differences ($P \leq 0.01$), the highest values were 72.24 mg/g in 100 mg/ml. Furthermore, the findings showed that when compound concentrations rose, the scavenging activity (antioxidant activity) gradually increased. The activity of coumarin gradually increases from 29.58 at 0.312 mg/ml to 63.50 at 10 mg/ml. The activity of the natural antioxidant (vitamin C) increased sharply at lower concentrations (from 72.72 at 0.312 mg/ml to 90.43 at 2.5 mg/ml), but at higher concentrations, the rate of increase slowed and eventually reached a constant value of 92.22 at 10 mg/ml. Similarly, the artificial antioxidant (BHT) increased in activity as concentration increased, starting at 32.66 at 0.312 mg/mL and reaching 90.12 at 10 mg/ml. The study's findings demonstrated that the coumarin blocked 100% of *S. aureus* biofilm development at 8 mg/ml and decreased it at 2 mg/ml. The investigation revealed that all isolates possessed the virulence genes *icaA* and *cifA*, which are in charge of biofilm formation in *S. aureus*. Additionally, compared to the isolates that were left untreated, the gene expression data revealed lower levels of *icaA* and *cifA* following coumarin treatment.

***Keywords*:** Anti-biofilm, Coumarin, *S. aureus*, *icaA*, *cifA*.

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Introduction

Gram-positive *Staphylococcus aureus* bacteria frequently live commensally on human skin and mucous membranes and are known to cause a variety of infectious illnesses, such as pneumonia, osteomyelitis, endocarditis, bacteremia, and skin abscesses (1). Numerous virulence factors, such as poisonous chemicals, bacterial adhesions that allow the bacteria to adhere to the surface of the

host extracellular matrix, and the capacity to withstand phagocytosis and biofilm formation, can be produced by *S. aureus* (2). Communities of microorganisms known as bacterial biofilms may adhere to a surface and go through significant modifications as they transform from planktonic to cell-like creatures. The phenotypic traits that biofilm bacteria have acquired in response to various environmental cues are indicative of these modifications (3). Many times, biofilm-associated bacterial infections are resistant to several antimicrobial tactics, mostly because they have a compact protective layer that shields them from the innate immune system and antibiotic therapy through restricted drug penetration (4).

The issue of *S. aureus* multi-drug resistance is severe. Individuals with diabetes, compromised immune systems, and compromised immune systems are more susceptible to *Staphylococcal* infections, especially those produced by *S. aureus*, which can result in infections of the skin and soft tissues. Toxins released by *S. aureus* overgrowth in an infected part of the body can cause toxic shock syndrome, a potentially fatal condition that makes *S. aureus* infections more severe **(5)**. More and more natural ingredients are being used in the drug-making process. In addition to being used as direct therapeutic pharmaceuticals, bioactive chemicals are also used as a primary ingredient in pharmaceutical manufacturing or as a building block for creating new, physiologically active compounds. Consequently, consideration was given to using natural remedies rather than medications and antibiotics (6). Recently, it has become more important to locate plants with significant antioxidant capacities since They might protect against free radicals and delay the development of several chronic illnesses (7). The coumarin class of natural phenolic compounds is distinguished by the presence of a single benzene bonded to a α-pyrone ring. It is notable for its strong biological potential, including antifungal, antibacterial, and antibiotic resistance modulating properties (8). With coumarin, one of the main polyphenols, acting as a model for the identification and examination of mechanisms of action, a number of bacterio-ecophysiological features also imply that plants may be a very helpful source of several more anti-biofilm chemicals (9).

Materials and Methods

Isolation of bacteria

The lab. of the UOB Institute of Genetic Eng. and Biotech.provided Twenty-five isolates of S. aureus.These isolates were previously obtained from wound specimens from hospitals in the city of Baghdad and were diagnosed through molecular and chemical testing. Ten isolates of S. aureus that were resistant to several drugs were chosen, By growing the isolates on Mannitol salt agar and applying the VITEK-2 technology, the diagnosis was verified. The isolates were re-cultured on the nutrient agar medium and then incubated aerobically for 24h at 37°C.

Assessment of biofilm formation

The technique described by Patel *et al*. (10) was used to quantify the formation of *S. aureus* biofilms; all isolates were grown for one night at 37 °C in Brain Heart Infusion Broth. Each isolate was carefully mixed with 1% glucose-containing tryptic soy broth (TSB) using pipetting. To satisfy the McFarland No. 0.5 turbidity criteria, the bacterial isolate suspension was modified.

A sterile, flat-bottomed 96-well microtiter plate was filled with Two hundred microliters of each isolate's

culture in triplicate. Following being covered with lids, the plates were incubated in an aerobic environment at 37°C for 24 hours. To get rid of any loose bacteria, Following incubation, the planktonic cells were washed twice with distilled water. Each well's bacterial cells were fixed for 20 minutes at room temperature in 200 µl of pure methanol. To stain the adherent cells, 200 µl of 0.1% crystal violet was added to each well and left for 15 minutes. After the staining procedure is finished, the excess stain was periodically washed away with distilled water two to three times. To remove the stain, 33% acetic acid was poured to the plate after it had been left at room temperature for around 30 minutes to ensure it was totally dry. An ELISA auto reader was used to measure optical density (O.D) at a wavelength of 630 nm. Three separate replications of each experiment were carried out in triplicate. In addition, a cut off value (O. D. c.) was established. It is defined as three standard deviations (S.D) above the mean O.D of the negative control: O. D. c. = average O.D of negative control + (3 × S.D of negative control). The isolates were classified into the four following categories based upon the OD: non-biofilm producer (O.D < O. D. c.); weak-biofilm producer (O. D. c. < O.D < 2 × O. D. c.); moderate-biofilm producer (2 × O. D. c. < O.D < 4 × O. D. c.); strong-biofilm producer (4 × O. D. c. < O.D) (11).

Determination of total phenolic content of Coumarin

The Folin-Ciocalteu technique, as reported by Jayaprakasha *et al*. (12), was used to spectrophotometrically measure the total phenolic content of coumarin. 0.4 ml of each sample was combined with 2.0 ml of the Folin-Ciocalteu reagent (diluted 10 times), and 1.6 millilitres of a sodium carbonate solution at 7.5%. To get the total down to 5 ml, distilled water was added. After covering the tubes with parafilm and letting them remain at room temperature for half an hour, the absorbance was measured spectrometerically at 760 nm.

A gallic acid calibration standard curve Figure 1, was used to determine the total phenolic content. The findings were expressed as milligrammes of gallic acid equivalent per gramme of dry weight.

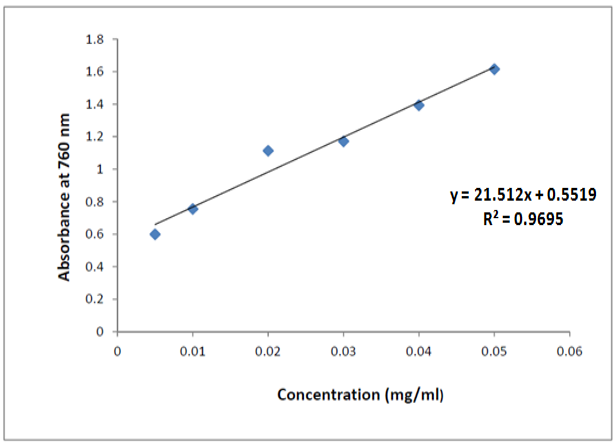

Figure (1): Standard curve of Gallic acid (13)

Evaluation of the antioxidant activity (DPPH assay)

According to Ogunmoyole *et al*. (14), the concentration of generated coumarin was examined for its antioxidant properties. Fifty microlitres of different doses (0.312, 0.625, 1.25, 2.5, 5, and 10) mg/ml were mixed with

five millilitres of freshly prepared 0.004% 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol. In order to create these concentrations, 0.1 grammes of the coumarin concentration extract were dissolved in distilled water. Then, by completing the volume into 10 ml, the working solution was created 10 mg/ml. Two successive dilutions of the coumarin concentration were performed to get 10-0.312 mg/ml.

After 30 minutes, each dilution's absorbance was measured at 517 nm. Vitamin C and butylated hydroxytoluene (BHT) served as positive controls. Every test was run in triplicate. The DPPH radical scavenging capability, also known as the % DPPH reduction, was computed as follows:

% Reduction = (Abs DPPH – Abs Dil.) /Abs DPPH x 100

Whereby:

Abs DPPH = average absorption of the DPPH solution, Abs Dil. = average absorption of the three absorption values of each dilution.

Microsoft Excel was used to create a visual utilising the numbers that were gathered. The image was used to determine each compound's EC_{50}, or the compound's effective concentration that reduced 50% of DPPH.

Study the antibiofilm activity of Coumarin

The anti-biofilm activity of coumarin concentration was assessed using a 96-well microtiter plate. The chemical was serially diluted twice on the plate to produce concentrations between 64 and 0.5 mg/ml. for the coumarin concentration, while the working solution was made at 128 mg/ml in broth. To the first wells in row A, 100 µl of the prepared coumarin concentration was added. Columns B–H contained just 100 µl of the broth. Methodically, twofold serial dilutions were made down the columns (from rows A-H) using a micropipette. Following the removal of 100 µl from the starting concentrations in row A, 100 µl of broth was added to the next row, well mixed, and the procedure was repeated until the last row (H), at which point the remaining 100 µl was discarded. Each well received 100µl of the 1×10^8 CFU/ml bacterial inoculum, with the exception of the negative control. It was done in the same way as described in the paragraph (Assessment of biofilm).

Polymerase Chain Reaction (PCR) for the molecular identification of virulence genes

Extraction of DNA

High-quality genomic DNA was exrtracted from both Gram-positive and Gram-negative bacteria with the EasyPure® Bacteria Genomic DNA kit (TRANS/China). DNA from the genome was extracted.

Molecular Detection of *clfA* and *icaA* genes

This phase involved adding 12.5 µl of OneTaq (NEB®) mastermix, 3 µl of DNA sample, 1 µl of 10 pmol/µl of each primer, and 7.5 µl of free-nuclease water, all while using the optimal PCR conditions for the gene.

Gene expression Analysis Using qRT PCR Technique

Measurements of the gene expression of *clfA* and *icaA* associated with biofilm formation were made to ascertain the effect of the coumarin concentration on these genes in the resistant isolates both before to and after treatment with the coumarin molecule. Following the manufacturer's recommended procedure, TRIzolTM Reagent was used to extract RNA. The primers used to measure the expression of the *clfA* and *icaA* genes are listed in Table 1, and a synopsis of the reaction mixture is given in Table 2. Furthermore, the heat cycler process was improved by a number of

experiments and is shown in Table 3. The results of the qRT-PCR analysis were computed by directly comparing the Ct values of the reference (housekeeping) gene and the target gene. The ΔΔCt was used to assess the relative levels of gene expression (fold change) in order to evaluate the genes (15).

Table (1): Primers utilized in this Study

Primer name		Sequence (5´-3´)	Size	Reference
icaA	F	**GAGGTAAAGCCAACGCACTC**	**151 bp**	**(16)**
	R	**CCTGTAACCGCACCAAGTTT**		
clfA	F	**ACCCAGGTTCAGATTCTGGCAGCG**	**165 bp**	**(16)**
	R	**TCGCTGAGTCGGAATCGCTTGCT**		
Housekeeping Gene *GyrB*	F	**GCGTCCGTTGATTGAAGCG**	**240 bp**	**(17)**
	R	**AACGTCACTTGCAACATCGC**		

Table (2): Volumes and concentrations of qRT-PCR reaction mix

Component	Volume (µl)
Luna Universal qPCR Master Mix	10
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
Template DNA	5
Nuclease-free Water	3
Total	**20**

Table (3): qRT-PCR Cycling Program

Cycle Step	Temperature	Time	Cycles No.
Initial Denaturation	95 °C	60 seconds	1
Denaturation	95 °C	15 seconds	40
Anneling	60 °C	30 seconds	
Melt Curve	60-95 °C	40 minutes	1

Results and Discussion

Detection of biofilm formation

The microtiter plate approach, which uses an ELISA reader to measure absorbance at 630 nm, is regarded as a standard test for the detection of quantitative biofilm biomass and is used to evaluate biofilm intensity (18). The isolates' mean optical density, which fell between 0.215 to 0.622, was 0.435 ± 0.287. As shown in Table 4, the results showed that all isolates exhibited 100% strong biofilm formation.

Staphylococcus aureus's capacity to produce an extracellular slime and a biofilm helps it withstand the human immune response and complicates clinical therapy since the biofilm protects the germs from antimicrobial drugs (19). Prior research showed a relationship between *S. aureus's* capacity to produce biofilms and its multi-drug resistance phenotype (20). The importance of biofilm and how it contributes to certain bacterial species' high levels of antibiotic resistance were emphasised by Bacalso *et al*. (21). The formation of a biofilm allows bacteria to stick to their host cells (22). Setiabudy *et al*. (23) and Ahmed *et al*. (24), who discovered that every *S. aureus* isolate developed a biofilm, are in agreement with the results of the current investigation.

Table (4): Biofilm forming of *S. aureus* isolates

Isolate	Biofilm formation	Isolate	Biofilm formation
***St*$_1$**	Strong	***St*$_6$**	Strong
***St*$_2$**	Strong	***St*$_7$**	Strong
***St*$_3$**	Strong	***St*$_8$**	Strong
***St*$_4$**	Strong	***St*$_9$**	Strong
***St*$_5$**	Strong	***St*$_{10}$**	Strong

(St): *Staphylococcus aureus*

Total phenolic content of Coumarin concentration

Many phenolic compounds have been investigated for their health advantages and biological characteristics (25). Folin-Ciocalteu reagent was therefore used to assess the coumarin's total phenolic content. The findings demonstrated a progressive rise in the coumarin concentration's total phenolic content (TPC) with concentration increases, with significant differences (P≤0.01). The highest results, as indicated in Table 5, were 72.24 mg/g in 100 mg/ml.

The findings of this investigation concurred with those of Siddiqui *et al*. (26), who demonstrated that the total phenol content in coumarin extracts was determined to be comparable to the phenol content at various concentrations, indicating an increase in content with increasing concentration. According to another study by Swaidan *et al*. (27), coumarin extracts have a notable phenol content. The total phenolic content of methanolic extracts from different plants on the Greek island of Crete was examined in a research by Kalpoutzakis *et al*. (28). The phenolic content was calculated using a gallic acid-based calibration technique and reported as mg GAE/g (gallic acid equivalents). The findings revealed that the phenolic content of the various plants varied significantly, ranging from 17.4 to 745.5 mg GAE/g. Coumarin molecules and their derivatives are crucial for disease prevention and therapy from a pharmacological perspective. Some are used as antibiotics, choleretic medications, anticoagulants, anticancer medicines, and antispasmodics. One such medication is novobiocin, which is a strong inhibitor of Gramme-positive bacteria (29).

Table (5): Total phenolic content of Coumarin

Concentration (mg/ml)	Mean ± SD
	Methanolic extract (mg/g)
100	72.24 ± 0.04
50	55.66 ± 0.04
25	28.17 ± 0.02
LSD value	0.100**
** (P≤0.01)	

Antioxidant activity of Coumarin concentration

Compounds' capacity to function as hydrogen donors or free radical scavengers has frequently been tested using the comparatively stable DPPH radical. Additionally, the antioxidant activity was assessed using this capacity (30). Coumarin's ability to scavenge radicals was evaluated at doses of 0.312, 0.625, 1.25, 2.5, 5, and 10 mg/ml. The controls were vitamin C and butylated hydroxy toluene (BHT).

The findings showed that when compound concentrations grew, the three compounds' scavenging ability (antioxidant activity) gradually increased as well. At 0.312 mg/ml, coumarin's activity gradually increases from 29.58 ± 0.02 to 63.50 ± 0.03 at 10 mg/ml. At lower concentrations, the natural antioxidant (vitamin C) showed a sharp increase in activity (from 72.72 ± 0.02 at 0.312 mg/ml to 90.43 ± 0.02 at 2.5 mg/ml). However, at higher concentrations, the rate of increase slowed and eventually reached a constant value of 92.22 ± 0.02 at 10 mg/ml. The artificial antioxidant (BHT) also showed an increase in activity with increasing concentration, beginning at 32.66 ± 0.02 at 0.312 mg/mL and

reaching 90.12 ± 0.01 at 10 mg/ml, According to Table 6.

Additionally, the half-maximal effective concentration (EC_{50}), which is the concentration of a medication that produces a reaction halfway between the baseline and maximum, is used in pharmacology to express a compound's potency. Since a compound's potency is inversely correlated with its EC_{50} value, and the most powerful molecule is the one with the lowest EC_{50}, it is paradoxical to represent a drug's potency by its EC_{50} value in the context of bioactivity-guided purification, even though it makes sense in a clinical setting (31). As seen in Figure 2, the radical scavenging capacity (EC_{50}) of the BHT was 0.2 mg/ml and that of the Coumarin was 5 mg/ml. However, the V.C. was significantly more effective than both of these substances, with an EC_{50} of 0.1 mg/ml.

Compounds with antioxidant activity are able to absorb free radicals, which are unstable chemicals that have the potential to harm cells. The chemicals' ability to shield cells from harm brought on by free radicals increases with concentration because of their increased antioxidant activity. Studies have demonstrated that coumarin's content increases its antioxidant activity, which increases its efficacy in minimising damage caused by free radicals. Coumarin is well-known for its antioxidant qualities (32).

Additionally, a study by Patel *et al*. (33) evaluated the free radical scavenging activity of three coumarin compound derivatives, I, II, and III, using ascorbic acid as a standard compound. They discovered that compound I and II exhibited strong antioxidant activity in comparison to ascorbic acid. Each coumarin compound's EC_{50} was determined, using ascorbic acid as a reference. As the amounts of the test substances grew, so did the scavenging effect. In comparison to ascorbic acid, which has an EC_{50} of 829.85 µM, coumarin compounds had EC_{50} values of 799.83 µM, 712.85 µM, and 872.97 µM for compounds I, II, and III, respectively.

According to a another study by Alshibl *et al*. (34), only compounds 3b and 5d exhibited moderate antioxidant activity among the pyranocoumarins, with corresponding EC_{50} values of 48.38 ± 4.61 and 82.92 ± 3.30µg/ml.

The concentration and mean values presented by this study are consistent with several reference studies that address the effects of coumarin, vitamin C, and BHT (butylated hydroxytoluene) on a variety of biological activities, including antibacterial characteristics. The antioxidant qualities of coumarin and its derivatives were assessed by Cheke *et al*. (35), who found that they were significantly active at different concentrations. Additionally, The antioxidant properties of coumarin, vitamin C, and BHT were compared by Qu *et al*. (36), who found that coumarin showed a dose-dependent increase in activity, with greater concentrations corresponding to higher mean values. According to Hadi *et al*. (37), coumarin compounds have antibacterial and antioxidant properties. They found that coumarin is more effective than vitamin C and BHT at doses ranging from 0.312 mg/ml to 10 mg/ml.

Table (6): Radical scavenging activity of coumarin

Concentration mg/ml	Mean ± SD			LSD value
	Coumarin	Vit. C	BHT	
10	63.50 ± 0.03	92.22 ± 0.02	90.12 ± 0.01	0.062**
5	50.24 ± 0.02	91.17 ± 0.03	82.56 ± 0.03	0.073**
2.5	42.83 ± 0.01	90.43 ± 0.02	76.36 ± 0.02	0.054**
1.25	38.65 ± 0.02	87.21 ± 0.01	68.72 ± 0.02	0.043**
0.625	31.38 ± 0.02	80.44 ± 0.02	45.78 ± 0.02	0.046**
0.312	29.58 ± 0.02	72.72 ± 0.02	32.66 ± 0.02	0.046**
LSD value	0.045**	0.044**	0.047**	----
** (P≤0.01)				

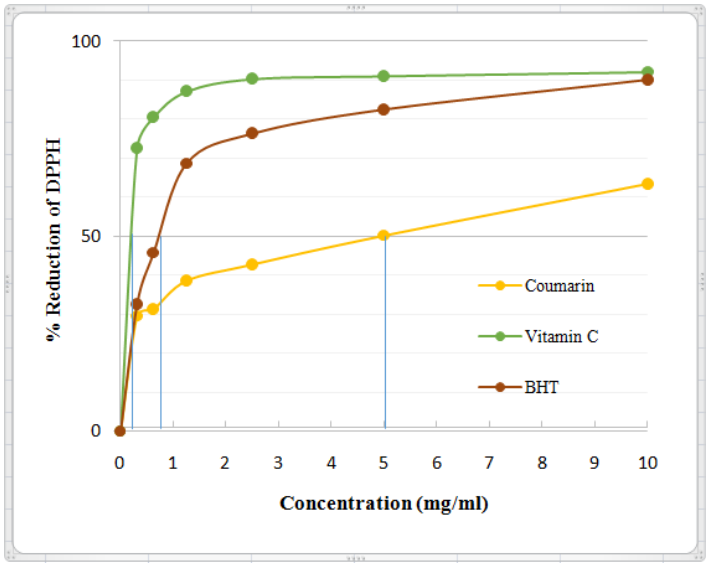

Figure (2): EC50 of coumarin

Anti-Biofilm activity of Coumarin

Staphylococcus aureus biofilm is one of the primary features of respiratory infections brought on by cystic fibrosis. These infections have become resistant to medications, leading to the emergence of methicillin-resistant *S. aureus* (MRSA) (38). Finding new and effective substances to stop the formation of bacterial biofilms is therefore essential. According to the study's findings, the coumarin decreased *S. aureus* biofilm development at 2 mg/ml and completely prevented it at 8 mg/ml, as indicated in Table 7.

Several techniques were employed to ascertain if bacteria formed biofilms, however the microtiter plate method is regarded as a standard test for biofilm biomass detection (18). Due to their ability to survive a range of stressors, avoid host defences, and acquire multidrug resistance, bacterial biofilms intricate structures made up of bacteria and other microorganisms become a major global health concern and contribute to chronic bacterial infections that affect humans and other organisms (39). The potential of coumarins as alternative treatment approaches has been emphasised in several papers due to their capacity to suppress biofilm formation in clinically relevant pathogens and impede QS signalling systems (40). According to Wahab *et al.* (41), the coumarin derivatives have

strong antibiofilm properties. Both the formation of MRSA biofilm and the breakdown of preexisting biofilm are effectively inhibited by these compounds.

Table (7): Biofilm formation of *S. aureus* isolates before and after treatment with Coumarin

No of isolates	Before treatment	After treatment concentration							
		0.5	1	2	4	8	16	32	64
St_1	Strong	Weak	Weak	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm	No Biofilm
St_2	Strong	Weak	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm	No Biofilm	No Biofilm
St_3	Strong	Moderate	Weak	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm	No Biofilm
St_4	Strong	Moderate	Moderate	Moderate	Moderate	No Biofilm	No Biofilm	No Biofilm	No Biofilm
St_5	Strong	Moderate	Moderate	Weak	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm
St_6	Strong	Moderate	Weak	Weak	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm
St_7	Strong	Moderate	Weak	Weak	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm
St_8	Strong	Strong	Moderate	Moderate	Moderate	No Biofilm	No Biofilm	No Biofilm	No Biofilm
St_9	Strong	Moderate	Weak	Weak	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm
St_{10}	Strong	Strong	Moderate	Weak	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm

(St): *S. aureus*, Control negative (cut off) = 0.13

Molecular detection of genes responsible for biofilm formation in *S. aureus*

Detection of *icaA* and *clfA* Genes

The *icaA* and *clfA* genes that cause *S. aureus* to generate biofilms were found in this work using the PCR method. When compared to the DNA ladder, the gel electrophoresis results revealed that the amplicons for *icaA* and *clfA* were 151 bp and 165 bp, respectively. The findings demonstrated that the *icaA* and *clfA* genes were present in all *S. aureus* isolates, as seen in Figures 3 and 4.

Among other sticky components, it was determined that the polysaccharide intercellular adhesin (PIA), encoded inside the ica operon, was crucial for the production of *staphylococcal* biofilms (42). The four gene operon (*ica* ABCD), which codes for the critical proteins required for PIA synthesis, makes up the locus of intercellular adhesion of *ica*. The production of exopolysaccharides is primarily regulated by the first two genes, *icaA* and *icaB* (43). The results of the present investigation agreed with those of Refaat *et al*. (44). Clinical *S. aureus* isolates showed varying capacities to produce biofilm, with the presence of the related genes indicating the roles for *icaA* genes as biofilm producer markers. The results of biofilm gene detection showed that 100% of isolates were positive for the *icaA*, *icaB*, and *icaD* genes and 56.25 percent for the *icaC* gene.

According to Abdel-Shafi *et al*. (45), a molecular screening method employing PCR amplification was used to examine 30 strong biofilm-forming isolates for the presence of the *icaA* or *icaB* genes. Of the 15 isolates under investigation, 9 had the *icaA* gene and 10 had the *icaB* gene. According to a study by Al-Muaala et al. (46), 27 (100%) *S. aureus* isolates had positive *clfA* gene tests. However, Al-Ezee *et al*. (47) discovered that 80% of isolates of *S. aureus* carried the *clfA* gene.

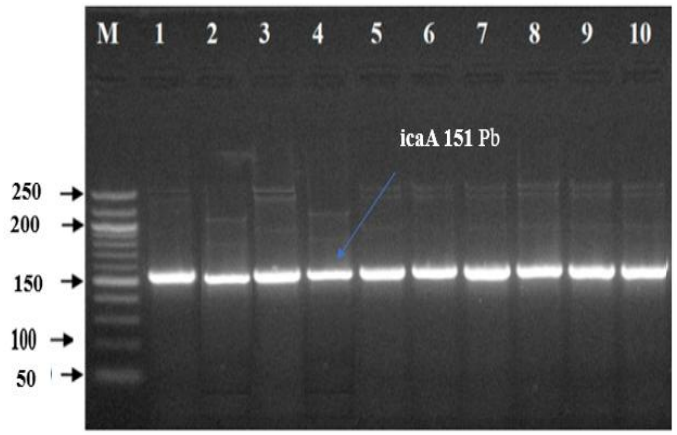

Figure (3): Gel electrophoresis of amplified *icaA* (151 bp), from *S. aureus* using conventional PCR. Agarose 2% stained with DNA loading dye. DNA ladders 50-250 bp and visualized on a UV transilluminator.

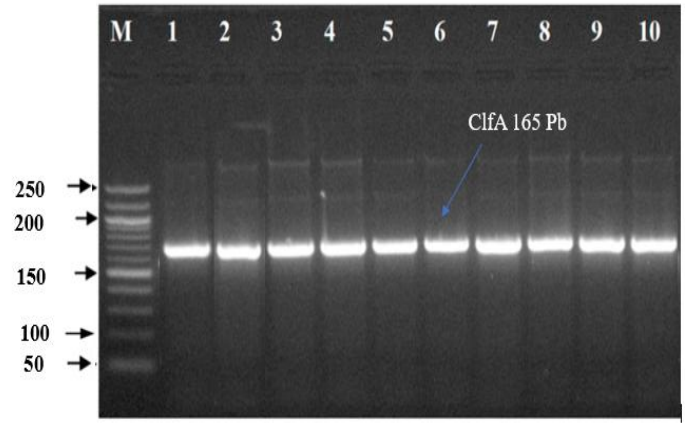

Figure (4): Gel electrophoresis of amplified *clfA* (165 bp), from *S. aureus* using conventional PCR. Agarose 2% stained with DNA loading dye. DNA ladders 50-250 bp and visualized on a UV transilluminator.

Gene expression of *icaA* and *clfA* genes

The quantitative RT-PCR test was used in this work to compare untreated isolates with isolates treated with coumarin in order to analyse the mRNA expression of the *icaA* and *clfA* genes Tables 8 and 9.

The chosen reference genes have a significant impact on the accuracy of qRT-PCR results (48), and their validity requires the appropriate application of qRT-PCR to investigate changes in target gene expression (49).

The amplification was recorded using the cycle threshold, or Ct value; low Ct values signify significant gene expression, whereas high Ct values signify low gene expression. The CT value rose in coumarin-treated isolates, suggesting that the *icaA* and *clfA* genes, which are in charge of *S. aureus* biofilm formation, are not highly expressed. According to Weerasekera *et al*. (50), biofilms are microbial cells that are adhered to the surface and incorporated in an extracellular matrix made of both host and microbial cells. Through a variety of processes, such as drug modification, neutralisation, hydrolysation, and efflux pump activation, biofilms exhibit enhanced resistance to antibiotics; in contrast, an extracellular matrix made up of polymeric materials functions as an external physical barrier and lowers the

permeability of antibiotics (39). Most human microbial illnesses are biofilm infections, and because biofilm is resistant, it is more challenging to treat and eradicate. Therefore, the development of new, highly efficient anti-biofilm agents is becoming essential.

To investigate their impact on gene expression, several researchers employed phenolic chemicals or plant extracts. El-Mahdy *et al*. (51) examined the effects of two natural polyphenols, curcumin and resveratrol, on lowering the production of toxin genes in MRSA isolates. Curcumin was shown to up-regulate the expression of certain isolates while down-regulating that of others. Furthermore, a research by Wang *et al*. (52) shown that following a 6 hour treatment with *Ginkgo biloba exocarp* extracts, the expression of *MRSA* biofilm-associated components *icaA* and *sarA* was decreased.

This study's findings were nearly identical to those of Wahab *et al*. (41). The quantitative RT-PCR study demonstrated the down-regulation of biofilm-associated genes, *icaA* and *icaD*, and the results demonstrated the strong anti-biofilm action of the coumarin derivatives. It was also shown that these coumarin derivatives did not cause fibroblasts to undergo cytotoxicity. Thus, the anti-biofilm capability of coumarin derivatives is identified in this investigation. According to El-Sawy *et al*. (53), coumarin exhibited antibacterial and anti-biofilm properties against *S. typhimurium*. By down-regulating biofilm regulatory genes, it suppressed the formation of cellulose and curli fimbriae, which in turn prevented the development of extracellular matrix.

Table (8): Gene expression for *icaA* gene before and after treatment with coumarin

Groups	samples	Ct reference gene	Ct target gene	CTΔ	CT ΔΔ	folding
Before treated (Control)	C1	25.3	14.06	-11.24	0	1
	C2	28.01	18.33	-9.68	0	1
	C3	22.03	10.13	-11.9	0	1
	C4	27.32	25.03	-2.29	0	1
	C5	26.01	22.96	-3.05	0	1
	C6	20.32	15.79	-4.53	0	1
	C7	21.82	22.08	0.26	0	1
	C8	19.45	14.59	-4.86	0	1
	C9	23.09	15.53	-7.5	0	1
	C10	23.10	19.67	-3.43	0	1
After treated with sub MIC of Coumarin	T1	10.88	13.96	3.08	14.32	0.000048
	T2	13.62	15.01	1.39	11.07	0.000465
	T3	19.03	15.13	-3.9	8	0.00390
	T4	21.49	24.73	3.24	5.53	0.02164
	T5	22.62	20.19	-2.43	0.62	0.6506
	T6	18.90	19.09.	0.19	4.72	0.03794
	T7	18.05	19.84	1.79	1.53	0.3462
	T8	20.54	19.90	-0.64	4.22	0.05366
	T9	27.09	19.97	-0.12	7.38	0.006003
	T10	25.99	23.54	-2.45	0.98	0.5069

Table (9): Gene expression for *clfA* gene before and after treatment with coumarin

Groups	samples	Ct reference gene	Ct target gene	CTΔ	ΔΔCT	folding
Before treated (Control)	C1	22.08	18.03	-4.05	0	1
	C2	25.33	15.30	-10.03	0	1
	C3	21.4	10.69	-10.71	0	1
	C4	20.19	19.44	-0.75	0	1
	C5	22.09	24.04	1.95	0	1
	C6	22.85	16.27	-6.58	0	1
	C7	17.56	16.49	-1.07	0	1
	C8	18.65	15.14	-3.51	0	1
	C9	30.09	21.17	-8.92	0	1
	C10	24.00	22.77	-1.23	0	1
After treated with sub MIC of Coumarin	T1	22.75	24.38	1.63	5.68	0.01950
	T2	23.02	13.64	-9.38	0.65	0.6372
	T3	18.01	15.71	-2.3	8.41	0.00293
	T4	21.2	22.08	0.88	1.63	0.3230
	T5	22.05	24.52	2.47	0.52	0.69737
	T6	22.11	16.71	-5.4	1.18	0.4413
	T7	20.15	19.12	-1.03	0.04	0.0972
	T8	23.53	24.38	0.85	4.36	0.04869
	T9	24.89	23.98	-3.91	5.01	0.0310
	T10	3300	21.09	-11.91	10.68	0.000609

Conclusion

Despite the fact that the bacterial isolates in this investigation produced substantial biofilms before treatment, the Coumarin compound demonstrated a high anti-biofilm agent on *S. aureus* and could down- expression the gene of *icaA* and *clfA* genes, which are important in biofilm formation in *S. aureus*.

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