



Effect of Norfloxacin and Curcumin on Genes Expression of *Staphylococcus aureus* isolated from clinical samples

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ABSTRACT

A total of 250 clinical specimens were collected from Baqubah Teaching Hospital and Al-Batoul Hospital in Diyala, Iraq, between December 2024 and February 2025. This study aimed to identify *Staphylococcus aureus* isolates using standard bacteriological methods, evaluate biofilm formation and efflux pump activity, and detect the *norB*, *fnbA* and *gyrB* genes molecularly. The effects of Norfloxacin and Curcumin on gene expression were also investigated. Among the collected samples, 25 isolates (10%) were identified as *S. aureus* from wounds, nasal swabs, ear infections, burns, and urine samples. Fifteen isolates (60%) were methicillin-sensitive *S. aureus* (MSSA), while 10 isolates (40%) were methicillin-resistant *S. aureus* (MRSA). Treatment with sub-MIC Curcumin significantly reduced biofilm formation and efflux pump activity. The findings also demonstrated significant reductions in the expression levels of *norB*, *gyrB* and *fnbA* genes after treatment with sub-MIC Curcumin, Norfloxacin, and their combination. The mean folding reduction values for these genes were (0.432, 1.175, 0.262), (0.263, 0.341, 0.039), and (0.547, 0.181, 0.009), respectively. These results suggest that Curcumin may act as an effective quorum sensing inhibitor against *S. aureus*.

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1. INTRODUCTION

Staphylococcus aureus a prominent pathogen that causes nosocomial and community-acquired infections in humans, a commensal Gram-positive cocci bacterium [1]. Moreover, it is linked to several human illnesses, such as skin infections and systemic ailments including sepsis, bacteremia, pneumonia, endocarditis, osteomyelitis, and toxic shock syndrome, making it a cause of recurrent and chronic infections [2].

Two important virulence mechanisms among several factors that enhance *S. aureus* pathogenicity are biofilm formation and efflux pumps. Biofilms protect bacterial colonies from medications and the immune system, hence promoting chronic infections [3]. Antibiotic resistance results from the active removal of drugs from bacterial cells by efflux pumps [4].

Plant-derived chemicals have special pharmacological properties, such as low cost, less toxicity, fewer side effects, and a decreased risk of developing resistance [5].

Ethical approval

The study was conducted following the ethical principles. It was carried out with patients' verbal and analytical approval before subjects were recruited for the study. The study protocol and the subject information and consent form were reviewed and approved by Diyala University, College of Sciences, Department of Biology approved this work. Research approval number 5031 in date: 8/12/2024.

2. METHODS

Isolation and identification of *S. aureus*

At the Baqubah and Al-Batoul Teaching Hospitals, two hundred fifty clinical Specimens were collected from different sources include burns, wounds, nose, ears, and urine between the beginning of December of 2024 to the February of 2025, all specimens were inoculated on blood agar and incubated at 37°C for 24 hours, *S. aureus* identified by morphological characteristics of colony and microscopic examination by gram staining to determine the arrangement, shape, stain reaction [6]. Catalase and coagulase biochemical's tests used as primary identification, developing colonies were cultivated for 24 hours at 37°C after being inoculated on mannitol salt agar to differentiate *S. aureus* from other *Staphylococcal* species. [7]. VITEK-2 Compact system Specimens for confirmation of identification [8]. To differentiate Methicillin resistance *S. aureus* Specimens (MRSA) from Methicillin sensitive *S. aureus* (MSSA), the Oxacillin disc diffusion method were depending, Oxacillin disc (5 µg) placed on the surface of Muller Hinton agar, then inoculated with test isolate, and incubated for 24 hours at 35°C. to determined the inhibition zone around the disc [9].

Determination of MIC for Curcumin and Norfloxacin

The broth microdilution method was used to determine the MIC of [10]. Serial concentration was prepared (2-1024µg/ml) for Curcumin and Norfloxacin. Twenty micro liters of bacterial suspension (1.5×10^8 CFU/ml) obtained by comparing overnight broth bacteria culture with 0.5 McFarland standard were added to each well. Twenty microliters of resazurin dye were applied and incubated for two hours following an 18–20-hour incubation period at 37°C. Bacterial growths was indicated by change in color from blue to pink. The lowest concentration that did not cause a color change was known as the MIC [11].

Biofilm Formation

The microtiter plate method was used to investigate the ability of isolate to form biofilm, bacterial suspension for each isolates were prepared after cultivating in nutrient broth for 24 hours at 37°C by comparing with to 0.5 McFarland (1.5×10^8 CFU/ml), 20µl of the suspension was added to 180µl of broth in microtiter plate wells. Following a 24-hour incubation period at 37°C, the wells were rinsed with saline, fixed for 15 minutes with 99% methanol, stained for 15 minutes with 1% crystal violet, and then rinsed with distilled water. 95% ethanol was used to extract the stain, and biofilm formation was assessed by observing the optical density at 630 nm. The values of the samples were subtracted from the OD value for the control well. The following is the outcome of comparing the cultivated pit's absorption to that of the control pits: If $OD \leq OD_c$ (seen as a non-biofilm producer); if $OD_c \leq OD \leq 2*OD_c$ (regarded as a moderate biofilm producer); and if $2*OD_c \leq OD$ (regarded as a strong biofilm producer). where OD s for the tested isolates and OD_c for the control pits. [12].

Efflux pumps production

S. aureus isolates' efflux pump activity was assessed using the Cartwheel technique [13]. 0.5 McFarland to 1µl was adjusted by overnight cultures after ethidium bromide concentrations of 1, 2, and 2.5 µg/ml were added to tryptic soy agar plates. The plates were then allowed to dry at room temperature before being examined under ultraviolet light with a UV transilluminator. Each isolate's capacity and ability to extrude ethidium bromide were evaluated.

Each isolate treatment with sub-MIC quantities of Curcumin by re- inoculate the plate by bacterial suspension incubate for 24 hours to evaluate the effect of sub-MIC Curcumin as natural quorum sensing inhibitor on efflux pump activity.

Molecular Study

Polymerase Chain Reaction (PCR) depend to detect the percentage of *S. aureus* genes (*gyrB* in quorum sensing, *fnbA* in biofilm, and *norB* in efflux pumps) for ten isolates.

DNA extraction: Using a commercially supplied genomic DNA purification kit provided by Promega (USA), genomic DNA was isolated from the bacterial isolates. and a nano-drop spectrophotometer was used to evaluate the purity of the purified DNA. After that, genomic DNA was measured and stored at -20°C. According to primer design (Table 1). The PCR protocol comprised a denaturation stage lasting five minutes at 95°C, one cycle of annealing at 54 to 58°C for sixty seconds, and a final extension at 72°C for five minutes at one cycle. The results of the PCR were found on gel electrophoresis.

[Table 1.](#) Primer sequence used for detection bacterial gene

Primer name	Sequence	Annealing temp C°	Reference
<i>norB</i>	F-5- AGCGCGTTGTCTATCTTTCC-3 R-5-GCAGGTGGTCTTGCTGATAA-3	60 C°	[14]
<i>gyrB</i>	F-5-CAAATGATCACAGCATTGTTGGTACAG-3 R-5-CGGCATCAGTCATAATGACGAT-3	54 C°	[15]
<i>fnbA</i>	F-5-CATAAATTGGGAGCAGCATCA-3 R-5-ATCAGCAGCTGAATTCCCAT-3	58 C°	[16]

Gene expression study by Real-time quantitative reverse transcription [qRT PCR]

This study used quantitative reverse transcriptase real-time qRT-PCR (Syber Green) to analyze gene expression of quorum sensing (*gyrB*), biofilm (*fnbA*), and efflux (*norB*) genes in three MRSA isolates St 3, St 5, St 9 after sub-MIC treatments of Norfloxacin, Curcumin, and their combination. Gene expression changes were measured by the comparative Ct method (2- $\Delta\Delta Ct$), with significant results defined as up-regulation (fold change > 1) or down regulation (fold change < 1). All target genes were successfully amplified in the analysis.

The RNA was extracted and purified from the sample using the TRIzol™ Reagent technique. A Quintus™ Fluorometer (Promega, USA) was used to measure the extracted RNA's concentration in compliance with the supplier's manual instructions. Real-time PCR was conducted using the Gene 9600 Quantitative Instrument. To determine the Cycle Threshold [CT] value by heat reaction, the primers that were prepared and the resultant solution were put into a real-time PCR cycler. RT-PCR is used to measure the expression levels of genes. The following measurements are made on a computer using the CT values that were measured during the heat reaction: The $\Delta\Delta Ct$ method was used to calculate the variations in gene expression levels in accordance with [17].

$$\Delta Ct(\text{test}) = Ct(\text{target}) - Ct(\text{internal control})$$

$$\Delta\Delta Ct = \Delta Ct(\text{test, treated}) - \Delta Ct_{\text{untreated}} = \text{ratio of normalized expression}$$

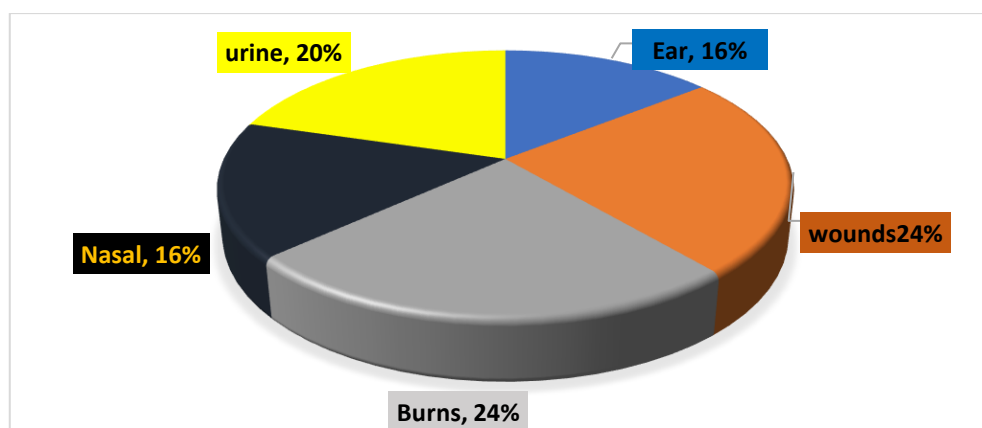
Analysis of statistics

The Statistical Packages of Social Sciences [18] program was used to assess the effects of various groups or factors in the research parameters.

3. RESULTS AND DISCUSSION

3.1 Isolation and identification of *S aureus*

Out of 250 total Specimens, 90 (36%) showed positive culture results. Among these, 25/250 (10%) were *S. aureus*, 6/25 (24%) for burn infections, 6/25 (24%) for surgical wounds, 5/25 (20%) for urine, 4/25 (16%) for ear and 4/25 (16 %) for nasal swabs, (Figure 1). The 25 isolates including 10 MRSA (40%) and 15 MSSA. (60%). This agreement with [19], who reported 61.81% MSSA and 38.18% MRSA. However, [20] found a higher MRSA rate (64.7%) and a lower *S. aureus* prevalence (26.56%). [21] reported 80 (72.7%), *S. aureus* isolates with 50 (62.5%) identified as MRSA.



[Figure 1.](#) Percentage of *S. aureus* according to source of specimens

All 250 blood specimens were cultured mostly on blood agar and incubated at 37°C for 24 hours. Gram stain investigation showed gram-positive cocci in each isolate, and the growing colonies of *S. aureus* were smooth, glistening, translucent, and clearly organized in a grape shape [22]. By digesting the mannitol sugar in the reddish agar and producing acids that lower the medium's pH and convert phenol red yellow, *S. aureus* was able to form yellow zones when expected bacterial colonies were subculture on mannitol salt agar medium [23].

The catalase test, gas bubble formation after adding 3% H₂O₂ to a colony indicated a positive result [24]. In the free coagulase test, equal volumes of bacterial suspension and citrated plasma were mixed and incubated at 37°C, clot formation confirmed *S. aureus* identification [25].

The results of biochemical tests, showed every isolate of *S. aureus* produced positive results for the coagulase test because it produces the coagulase enzyme [26] and the catalase test because the bacteria can produce the catalase enzyme [27]. All isolates were identified using the viteik-2 compact system.

3.2 Minimum inhibitory concentration (MIC) of Curcumin and Norfloxacin

The results in Table 2 showed that MIC ug /ml of Curcumin higher in MRSA isolates than MSSA where the values rang of curcumin for 10 MRSA isolates (16- 512 ug /ml) and (16–64 µg/ml) for MSSA isolates, because of its well-known anti-inflammatory and efflux pump inhibitory properties, Curcumin may be employed as an adjuvant instead of a main antibiotic. The multidrug resistance pattern of MRSA lessens its vulnerability to a wide range of widely used medications. According to earlier studies, Curcumin exhibits modest antibacterial activity against *S. aureus*, with MICs varying between 50 and 500 µg/ml, contingent on the experimental setup and bacterial strain [28].

The MIC values of norfloxacin were lower for MRSA isolates (16–128) and MSSA isolates (32–256) µg/ml. Its lower MIC values show that norfloxacin is more effective against bacteria than curcumin.

This result was also in line with a study by Al-Marjani [29] that was carried out in Pakistan and found that the MIC of *S. aureus* isolates for norfloxacin ranged from 32 to 1024 µg/ml. In addition, his results were different from a local investigation by Al-Marjani *et al.*, [30], which indicated that the range of norfloxacin's minimum inhibitory concentrations (MICs) was 4–1024 µg/ml, (Table 2).

Table 2. Curcumin and Norfloxacin minimum inhibitory concentrations (MIC) for *S. aureus*.

MIC ug/ml	Curcumin		Norfloxacin	
	MRSA	MSSA	MRSA	MSSA
16	1	8	3	1
32	1	4	3	6
64	0	3	2	4
128	2	00	2	3
256	2	00	00	1
512	4	00	00	00
Total	10	15	10	15

3.3 Formation of Biofilms

Twenty-five clinical isolates of *S. aureus* were evaluated in this investigation. 93% (14/15) of the MSSA group (n=15) formed strong biofilms, whereas 7% (1/15) formed moderate biofilms. Weak biofilm producers did not show up. All isolates (100%) in the MRSA group (n=10) had strong degree for biofilm formation, (Table 3). These results agreement with those of [31], who also noted that all *S. aureus* isolates produced biofilms, however, they do not agree with [32], who reported that only 79.6% of isolates were able to produce biofilms.

Table 3. Percentage of Biofilm formation for MRSA and MSSA.

MRSA			MSSA		
O.D.C = 0.092nm			O.D.C = 0.092nm		
Biofilm degree	No.	%	Biofilm degree	No.	%
Strong O.D =0.817- 1.607nm	10	100	Strong O.D =0.393- 1.373nm	14	93
Moderate	0	00	Moderate O.D =0.230 nm	1	7
Total	10	100	Total	15	100

3.4 Efflux pump production

The current investigation showed that all the 25 isolates in the two groups (15 MSSA and 10 MRSA) were positive for Efflux pump production all Et. Br concentrations (Figure 2). Our findings different from those of [33], who stated that the EtBr-agar cartwheel method achieved a positive result of 24.49 percent.

**Figure 2.** Efflux pumps production by *S. aureus* (EtBr-agar cartwheel method).

3.5 Effect of sub-MIC Curcumin on efflux pumps

MRSA isolates generally showed higher MIC of Curcumin values (16-512) $\mu\text{g/ml}$, and (16-64) $\mu\text{g/ml}$ for MSSA isolates. Similarly, past investigations have shown that Curcumin exhibits modest antibacterial activity against *S. aureus*, with MICs varying from 50 to 500 $\mu\text{g/ml}$, dependent on the experimental setting and bacterial strain [34,35].

Following treatment by sub-MIC of Curcumin for each *S. aureus* isolate, the results showed that all MSSA isolates not produce efflux pumps in all Ethidium bromide concentration whereas MRSA isolates showed reduction in efflux production from 10(100%) to 6 isolates only (60%). The changes in efflux activity before and after treatment were statistically significant (P-values 0.0001), (Table 4).

Table 4. Effect of sub-Mic Curcumin on Efflux pumps in *S. aureus*

Ethidium bromide concentration	Before treatment		After treatment	
	No (%)		No (%)	
	MRSA	MSSA	MRSA	MSSA
1 $\mu\text{g/ml}$	10(100)	15 (100)	1 (10)	0(0)
2 $\mu\text{g/ml}$	10(100)	15 (100)	2(20)	0(0)
2.5 $\mu\text{g/ml}$	10(100)	15 (100)	3(30)	0(0)
Total	10(100)	15 (100)	6 (60)	0(0)
P-value	0.0001 **		** (P $\leq$ 0.01)	

3.6 Molecular Detection of (*gyrB-fnbB-norB*) genes

Results of molecular analysis one quorum sensing gene, one biofilm formation gene, and one efflux pump gene in 10 clinical isolates of *S. aureus*. The genetic study contributes to conventional detection and investigation techniques with polymerase chain reaction (PCR), an effective tool in clinical microbiology investigations that has been widely utilized to identify bacteria and genes of interest [36].

The results of the current study's gene detection revealed that 8 (80%) isolates carried the *gyrB* gene (Figure 3). The *gyrB* gene in *S. aureus* encodes the B subunit of DNA gyrase, an enzyme that introduces negative supercoils into DNA and is essential for DNA replication, transcription, and recombination. Resistance to antibiotics like novobiocin and fluoroquinolones is linked to mutations in this gene. Found that novobiocin exposure induced *gyrB* mutations, which affected bacterial proliferation, stress survival, and cell wall permeability. These mutations also triggered compensatory metabolic changes impacting DNA replication and protein synthesis [37].

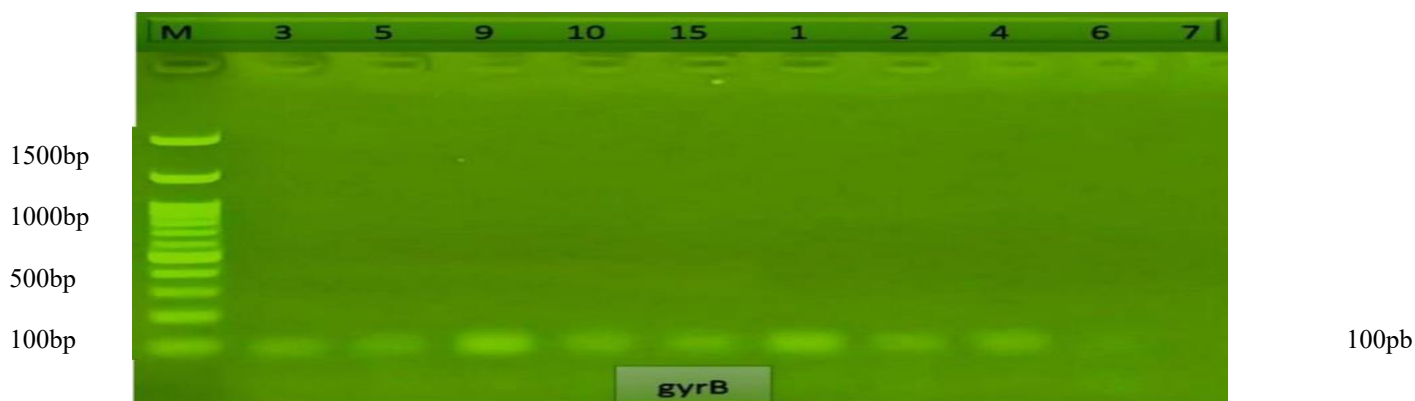


Figure 3. Result of the amplification of *gyrB* gene of *S. aureus* were fractionates on 1.5% Agarose gel electrophoresis. M:100 bp DNA Ladder marker, lanes MRSA (3,5,9,10,15) and MSSA (1,2,4,6,7) resemble 100 bp PCR products.

Isolates of *S. aureus* seem to have developed biofilms demonstrate the presence of the biofilm gene (*fnbA*) in *S. aureus* as well as the relationship between these genes. The current study's gene identification results showed that five isolates (50%) possessed the *fnbA* gene, (Figure 4). In comparison, 92% of the *fnbA* gene was present in [38].

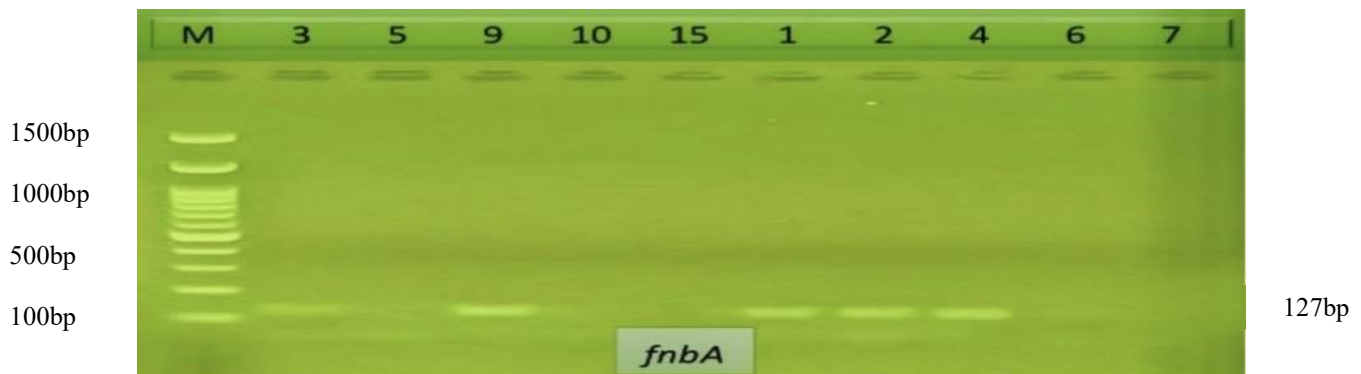


Figure 4. Agarose gel electrophoresis (1.5% agarose) for *fnbB* gene (524 bp amplicon) MRSA (3,5,9,10,15) and MSSA (1,2,4,6,7), lane 100 bp DNA Ladder.

As a marker for efflux pump genes, the *norB* gene typically indicates potential resistance to the fluoroquinolone group. The *norB* findings (100%) (Figure 5) were consistent with those of which reported that 98.2% of isolates contained *norB*[39]. However, this results contradicted studies conducted in China by [40,41] where two Iranian research efforts found *norB* findings of 83% and 41.75%, respectively.

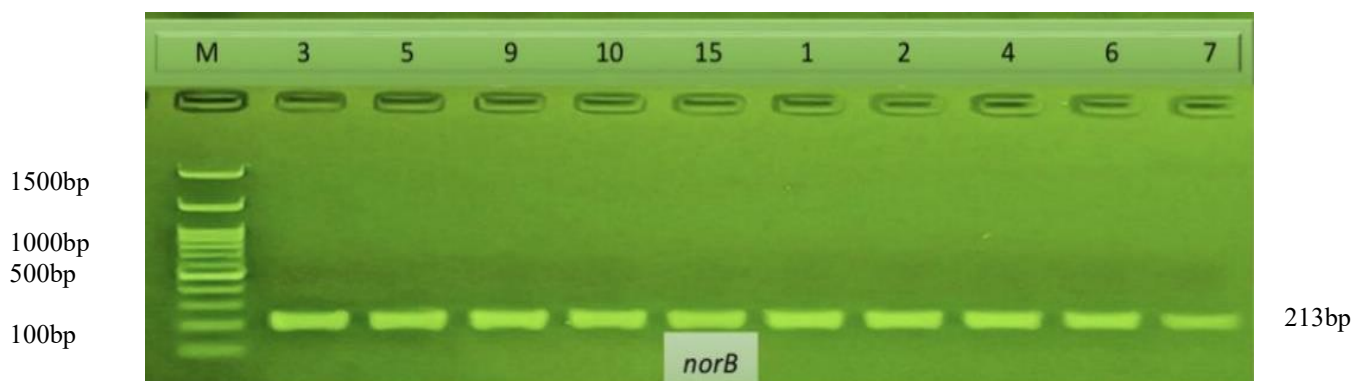


Figure 5. Agarose gel electrophoresis (1.5% agarose) for *norB* gene (213 bp amplicon) MRSA (3,5,9,10,15) and MSSA (1,2,4,6,7), lane 100 bp DNA Ladder.

3.6 Gene expression study by Real-time quantitative reverse transcription [qRT PCR]

3.6.1 The expression of quorum sensing gene - *gyrB*-

Isolates before and after treatment with Curcumin, Norfloxacin, and their combination using Ct values. Before treatment, Ct values ranged from 24.00 to 26.27. After treatment, Ct values decreased, indicating changes in gene expression. The fold values change after treatment were 1.175 for Curcumin, 0.341 for Norfloxacin, and 0.181 for their combination, showing significant down regulation, especially with the combined treatment, (Table 5). These results suggest that combining Curcumin and Norfloxacin effectively reduces *gyrB* expression as indicated in may help decrease antibiotic resistance. The p-value (0.0083) result showed a high significant differences between folding mean before and after treatment with the two agents and their mixture. Norfloxacin and plant extract together were found to be substantially more effective than either drug alone at lowering gene expression, enhancing the drugs' ability to kill bacteria, or creating a synergistic effect that outperformed antibiotics when taken alone [42].

[Table 5.](#) The treatments effect on expression of (QS) gene *gyrB*.

Isolate	16S	<i>gyrB</i>	ΔCT	ΔΔCT	Folding	Mean	Folding VS Control
St3	21.96	26.27	4.31	0.76	0.592	1.104	1
St5	21.26	24.00	2.74	-0.81	1.757		
St9	21.19	24.80	3.61	0.06	0.961		
St3(C)	16.80	22.73	5.93	2.38	0.193	1.297	1.175
St5(C)	18.86	21.43	2.57	-0.98	1.977		
St9(C)	18.49	21.26	2.77	-0.78	1.721		
St3(NOR)	17.79	24.93	7.14	3.59	0.083	0.376	0.341
St5(NOR)	16.16	19.80	3.64	0.09	0.942		
St9(NOR)	14.30	21.13	6.83	3.28	0.103		
St3(C+NOR)	14.32	19.18	4.86	1.31	0.404	0.200	0.181
St5(C+NOR)	13.92	20.94	7.02	3.47	0.090		
St9(C+NOR)	13.24	20.06	6.82	3.27	0.104		
P-value	0.0083**					**(P≤0.01)	
St = <i>S. aureus</i> , NOR = Norfloxacin, C = Curcumin							

3.6.2 The expression of Biofilm gene - *fnbA*-

The expression of the *fnbA* gene was examined both before and after curcumin, norfloxacin, and their combination treatments. After treatments, the initial range of Ct values, which was 23.98 to 29.91, shifted. Particularly with the combined treatment, the fold change values were 0.262 for Curcumin, 0.039 for Norfloxacin, and 0.009 for their combination, indicating considerable down-regulation, (Table 6). This drop in *fnbA* expression contributes to a decrease in biofilm development, and the p-value (0.0001) result revealed a highly significant shift. By deactivating bacterial adhesins and interfering with cell aggregation. *S. aureus* and *C. albicans* were reported to be resistant to curcumin and imidazole in biofilms, however, the number of living cells in the biofilms was dramatically reduced when these agents were coupled with antibiotics [43].

[Table 6.](#) The treatments effect on expression of biofilm gene *fnbA*.

Isola	16S	<i>fnbA</i>	ΔCT	ΔΔCT	Folding	Mean	Folding VS Control
St3	21.96	27.93	5.97	0.17	0.891	3.166	1
St5	21.26	23.98	2.72	-3.08	8.476		
St9	21.19	29.91	8.72	2.92	0.132		
St3(C)	16.80	25.06	8.26	2.46	0.182	0.828	0.262
St5(C)	18.86	45.00	26.14	20.34	0.000		
St9(C)	18.49	23.09	4.60	-1.20	2.303		
St3(NOR)	17.79	27.51	9.72	3.92	0.066	0.125	0.039
St5(NOR)	16.16	23.96	7.80	2.00	0.251		
St9(NOR)	14.30	24.21	9.91	4.11	0.058		
St3(C+NOR)	14.32	26.54	12.22	6.42	0.012	0.028	0.009
St5(C+NOR)	13.92	24.41	10.49	4.69	0.039		
St9(C+NOR)	13.24	23.90	10.66	4.86	0.035		
P-value	0.0001**					**(P≤0.01)	
St = <i>S. aureus</i> , NOR = Norfloxacin, C = Curcumin							

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3.6.3 The expression of *norB* Gene

The study measured *norB* gene expression before and after treatment with Curcumin, Norfloxacin, and their combination. Initial Ct values ranged from 25.24 to 28.61 and changed after treatments. Fold change values were 0.432 for Curcumin, 0.263 for Norfloxacin, and 0.567 for their combination, indicating a moderate decrease in *norB* expression. The changes in p-value (0.0288) were statistically significant, showing that these treatments reduce *norB* gene expression in (Table 7).

Zingiber officinale plant extract inhibits *acrAB*-induced efflux pump activity in *S. typhimurium* in vitro. Additionally, the plant extract is safe and effective in treating clinical patients with *S. typhimurium*, according to docking studies [44].

Table 7. The treatments effect on expression of (*norB*) gene

Isolate	16S	<i>norB</i>	Δ CT	$\Delta\Delta$ CT	Folding	Mean	Folding VS Control
St3	21.96	28.61	6.65	1.72	0.304	1.310	1
St5	21.26	25.36	4.10	-0.83	1.782		
St9	21.19	25.24	4.05	-0.88	1.845		
St3(C)	16.80	22.53	5.73	0.80	0.576	0.566	0.432
St5(C)	18.86	25.19	6.33	1.40	0.380		
St9(C)	18.49	23.85	5.36	0.43	0.744		
St3(NOR)	17.79	25.90	8.11	3.18	0.111	0.346	0.263
St5(NOR)	16.16	21.87	5.71	0.78	0.584		
St9(NOR)	14.30	20.78	6.48	1.55	0.342		
St3(C+NOR)	14.32	19.24	4.92	-0.01	1.009	0.716	0.567
St5(C+NOR)	13.92	20.1	6.18	1.25	0.421		
St9(C+NOR)	13.24	18.65	5.41	0.48	0.719		
P value		0.0288*					*(P≤0.05)

St = *S. aureus*, NOR = Norfloxacin, C = Curcumin

4. CONCLUSIONS

According to this study, *S. aureus* virulence factors such as biofilm formation, and efflux pump function are significantly inhibited by sub-inhibitory doses of Curcumin, Norfloxacin, and combination.

After three resistant isolates were administered sub-MICs of Curcumin, a natural quorum sensing inhibitor, the gene expression of *gyrB*, *fnbA*, and *norB* dropped more precipitously when Curcumin and Norfloxacin were combined. This implies a synergistic effect that significantly increased MRSA's susceptibility to different medications.

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