


# Antibacterial, Antibiofilm, and Anti-Quorum Sensing Potential of Novel Synthetic Compounds Against Pathogenic Bacteria Isolated From Chronic Sinusitis Patients

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## Abstract

Quorum sensing (QS) is a major controller of virulence and biofilm formation in pathogenic bacteria. The aim of the research was to screen novel synthetic compounds (18) from 2 series (Pyrazole and Diene dione) for quorum sensing and biofilm inhibitory potential against resistant pathogens isolated from patients with chronic sinusitis. Most of the compounds have documented zone of inhibition against Gram positive strains *Staphylococcus aureus*, *Enterococcus faecalis* and moderate activity against Gram negative *Klebsiella pneumoniae* and *Proteus mirabilis* in comparison with standard antibiotic. Compounds Q1 and Q7 have given the maximum zone of inhibition 18 and 20 mm with MICs 0.312 mg/mL and .156 mg/mL against *S aureus* and *E faecalis*, respectively. Some compounds were equally potent at inhibiting the formation of biofilm which later established by phase contrast microscopy. Regarding quorum sensing inhibition, the tested concentration of synthetic compound UA3 0.313 mg/mL inhibited violacein production without decreasing *Chromobacterium pseudoviolaceum* count which was significantly lower than determined MIC's. It was depicted from the results that selected compounds exhibited low level of cytotoxicity toward human red blood cells. Hence, these findings revealed that most novel compounds were effective antibacterial, whereas compound UA3 has shared significant anti-quorum sensing potential against *Chromobacterium pseudoviolaceum*.

## Keywords

quorum sensing, antibacterial potential, biofilm inhibition, synthetic compounds, sinusitis isolates

## Introduction

Antimicrobial is a substance which can efficiently inhibit growth of pathogens. These have been in use for the management of infectious diseases since the start of 20<sup>th</sup> century.<sup>1</sup> After the invention of penicillin, a list of antimicrobials was developed and many common bacterial diseases could be cured. The overuse of antibiotics in clinical practice resulted in emergence of drug resistance.<sup>2</sup> In Pakistan, multi drug resistance in *salmonella typhi* has increased due to which first line of defense antibiotics were become ineffective and drug of choice become third generation cephalosporin ceftriaxone.<sup>3</sup> The United States center for disease control and prevention (CDC) has found that microbial resistance cause over 2 million ailments and over 23,000 deaths annually, and this

number is going to increase in future.<sup>4</sup> The development of drug has usually focused on bacteria in planktonic forms. Many antimicrobials were initially developed to target individual microbe, being evaluated in vitro against microorganism growing in planktonic mode of life. But toward the end

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of the 20<sup>th</sup> century, it was clear that most bacteria survive in vivo in the form of more composite communities called biofilm. Biofilm is a mode of resistance bacteria develop against environmental stress, immune response, and antibiotics.<sup>5</sup>

Biofilm antibiotic tolerance is a capability of microbes living inside the biofilm to outlive antibiotic treatment using a certain genes. It is mostly natural form of microbial life because it increases its ability to fight against the hostile environmental conditions by avoiding the free flow of blood and water. Microbes residing inside biofilm are about 1000 times more resistant to antimicrobial that are in free floating form. Similarly, bacteria in deeper layers are also more protected from antibiotics and due to increase in cell number, providing an opportunity to transfer plasmid DNA carrying resistant gene through conjugation. Hence, the resistance due to biofilm is different from resistant phenomenon that operates at cellular level and cannot overlook in the development of new strategies to fight against infectious ailments.<sup>6</sup>

Quorum sensing system (QS) is a telephonic system in microbes which is a great regulator of bacterial functions. The QS controls the formation of biofilm, formation of virulence factor, and process of infection. Resistance to antimicrobial drugs has led to the inhibition of bacterial virulence factors, which are control by QS. Virulence factors comprised of adhesion, toxins, and some secretions.<sup>7</sup> Quorum sensing inhibitor impedes the virulence factors responsible for bacterial pathogenesis. The *chromobacterium pseudoviolaceum* is a Gram negative bacterium mainly present as harmless saprophyte and known as biosensor strain for quorum sensing inhibition. It produces quorum sensing signal molecule acyl homoserine lactone (AHL) which is involved in production of violacein.<sup>8</sup> QS inhibitor are a newly develop therapeutic agents which effectively control the virulence of bacteria and hence inhibit the growth of biofilm which make host to eliminate bacteria innately as well as allowing antibiotics to effectively kill the bacteria.<sup>9</sup>

Heterocyclic compounds are an extremely important and special class of compounds; they represent more than half of organic compounds having a broad range of biological activities. Thus, the basic structure of these compounds provides a structure on which different functional groups are arranged to produce an effective compound. Among heterocyclic compounds, nitrogen containing pyrazole compounds display potent biological and pharmacological properties.<sup>10</sup> Pyrazole compound have demonstrated excellent biological activities like analgesic, anti-inflammatory, antibacterial, antifungal, antiviral, and antitumor.<sup>11</sup> Dione-based thiosemicarbazide derivatives exhibited appreciable biological activity such as antifungal, antiviral, anticonvulsant, antimicrobial, and anti-Alzheimer. Indoline-2,3-dione derivatives document good antimicrobial activity, as it acts on many targets possess a wide spectrum of biological and pharmacological activities.<sup>12</sup>

The wide spread of multidrug-resistant strains (MDR), day by day, are becoming more problematic for human health and searching of new synthetic compounds become mandatory for successful treatment of bacterial diseases. Therefore, the

present study is planned to evaluate the antibacterial, biofilm, and quorum sensing inhibitory potential of pyrazole, diene, and dione derivatives.

## Materials and Methods

### Synthetic Compounds

Two series of synthetic compounds (Table 1) have been collected from Organic Chemistry Lab, Department of Chemistry, University of Education, Lahore, Pakistan. Synthetic compounds prepared (10 mg/mL) in dimethyl sulfoxide (DMSO). This concentration of sample solution was stored for use for the rest of the study.

### Test Organisms

Four characterized bacterial strains (*Enterococcus faecalis*, *Staphylococcus aureus*, *Proteus mirabilis*, and *Klebsiella pneumoniae*) isolated and characterized by conventional as well as molecular techniques from patients with chronic sinusitis have been collected from Medicinal Biochemistry Lab, Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan. All bacterial cultures grown for 24 h were adjusted to McFarland standard .5 equivalent to  $1.5 \times 10^8$  cfu/mL (*S. aureus*),  $1.9 \times 10^8$  cfu/mL (*E. faecalis*),  $2.1 \times 10^8$  cfu/mL (*K. pneumoniae*),  $1.6 \times 10^8$  cfu/mL (*P. mirabilis*).

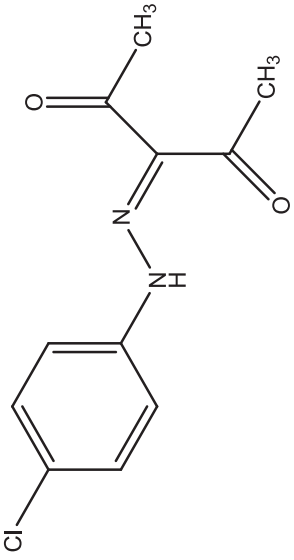
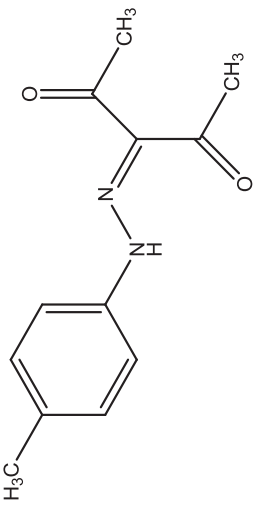
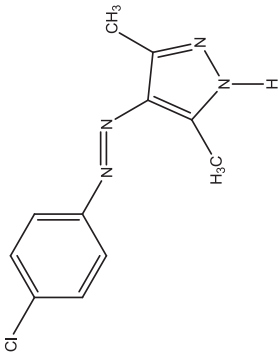
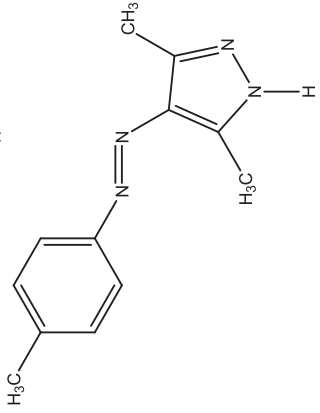
### Antibacterial Activity of Synthetic Compounds

Antibacterial activity of synthetic compounds was evaluated by well diffusion assay.<sup>13</sup> After preparing nutrient agar plates, refreshed bacterial culture was poured onto the plates. Wells (6 mm) were made with the help of sterilized borer. Samples in concentration of 10 mg/mL (100  $\mu$ L) were filled in these wells. Antibiotic (ciprofloxacin 10 mg/mL) was used as standard. Then, plates were incubated (Bolton incubator Model BS-C270) for 24 h at 37°C. Zone of inhibition was measured in millimeter (mm).

### Minimum Inhibitory Concentration

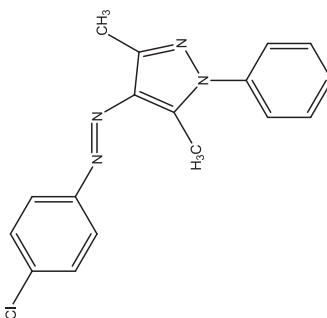
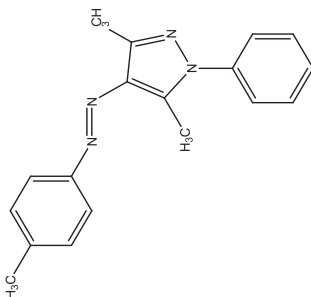
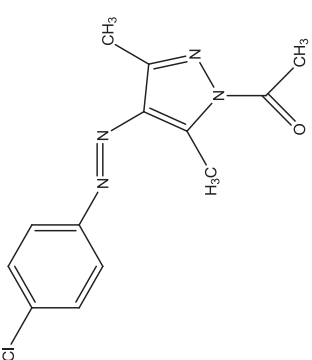
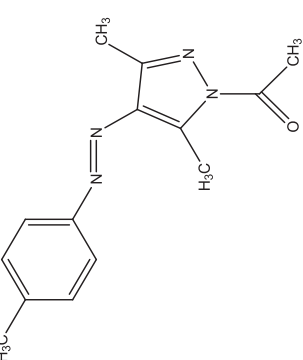
The resazurin-based micro-dilution assay was performed to evaluate the inhibitory effect of synthetic compounds against selected bacterial strains. Samples (10 mg/mL) (100  $\mu$ L) were put in the first row of 96-well plates. All other wells were filled with 50  $\mu$ L of Mueller Hilton broth (Oxoid UK). Micro-dilution of Compounds was carried out exactly according to laboratory standard protocol (CLSI). To each well of the plate (10  $\mu$ L), resazurin indicator solution (270 mg/40 mL of distilled water) was added and finally at the end bacterial culture (10  $\mu$ L) was added to each well of the plate. Each plate has negative (bacteria culture plus broth) and positive control (ciprofloxacin 10 mg/mL). Plates were incubated at 37°C for 24 h. The color change was noted visually. Bacterial growth

**Table 1.** Structural, Molecular Weight, and Molecular Formula of Synthetic Compounds.

Compound code	Structure	Soly.	Mol. Wt. g/mol	Mol. Formula
Q-3A		DCM	238.67	$C_{11}H_{11}ClN_2O_2$
Q-3B		DCM	218.25	$C_{11}H_{11}N_2O_2$
Q-5A		DCM	234.68	$C_{11}H_{11}ClN_4$
Q-5B		DCM	214.27	$C_{12}H_{14}N_4$

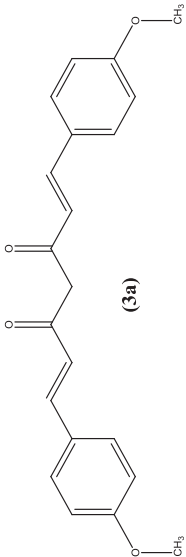
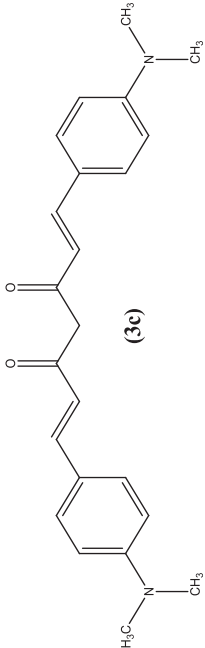
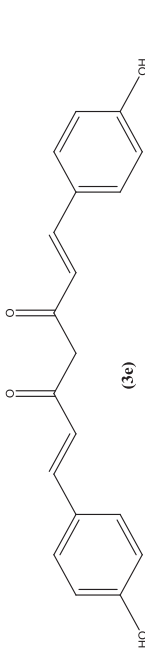
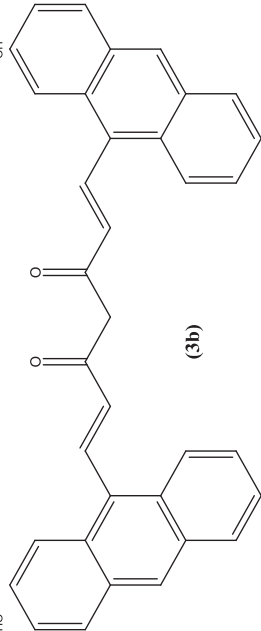
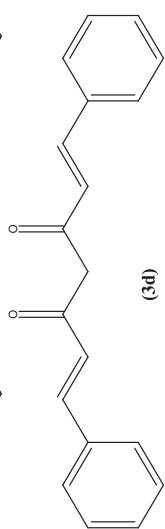
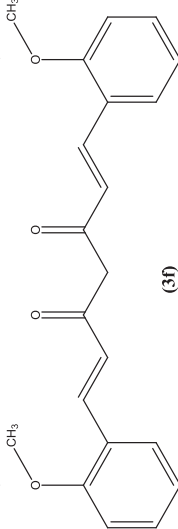
(continued)

Table I. (continued)

Compound code	Structure	Soly.	Mol. Wt. g/mol	Mol. Formula
Q-7A		DCM	310.78	C <sub>17</sub> H <sub>15</sub> ClN <sub>4</sub>
Q-7B		DCM	290.36	C <sub>18</sub> H <sub>18</sub> N <sub>4</sub>
Q-9A		DCM	276.72	C <sub>13</sub> H <sub>13</sub> ClN <sub>4</sub> O
Q-9B		DCM	256.30	C <sub>14</sub> H <sub>16</sub> N <sub>4</sub> O

(continued)

Table I. (continued)

Compound code	Structure	Soly.	Mol. Wt. g/mol	Mol. Formula
M-1	 (3a)	DCM	336.39	C <sub>21</sub> H <sub>20</sub> O <sub>4</sub>
M-2	 (3c)	DCM	418.19	C <sub>23</sub> H <sub>26</sub> N <sub>2</sub> O <sub>2</sub>
UA-4	 (3e)	DCM	308.33	C <sub>19</sub> H <sub>16</sub> O <sub>4</sub>
UA-6	 (3b)	DCM	476.18	C <sub>35</sub> H <sub>24</sub> O <sub>2</sub>
M-5	 (3d)	DCM	276.34	C <sub>19</sub> H <sub>16</sub> O <sub>2</sub>
UA-5	 (3f)	DCM	336.39	C <sub>21</sub> H <sub>20</sub> O <sub>4</sub>

(continued)

Table 1. (continued)

Compound code	Structure	Soly.	Mol. Wt. g/mol	Mol. Formula
UA-3	<p>(3g)</p>	DCM	366.33	$C_{19}H_{14}N_2O_6$
M-8	<p>(5)</p>	DCM	392.59	$C_{25}H_{36}N_4$
M-9		DCM	366.09	$C_{19}H_{14}N_2O_6$
UA-1		DCM	368.38	$C_{21}H_{20}O_6$

resulted in conversion of blue color to pink. The lowest concentration of compound which prevented the change in color from blue to pink indicates the MIC of compounds. The test was performed in triplicate.<sup>14</sup>

### Anti-Biofilm Potential

Biofilm inhibition potential of synthetic compounds was determined by using 4 bacterial strains *S aureus*, *E faecalis*, *P mirabilis* and *K pneumoniae*. To carry out biofilm inhibition assay, biofilms were grown on the 96-well plate.<sup>15</sup> Biofilm were subjected to inhibition by synthetic and standard antibiotic (Ciprofloxacin). The wells of micro-titer plate were filled with 100  $\mu$ L of nutrient broth (Oxoid-UK). Synthetic compound samples (10 mg/mL DMSO) 100  $\mu$ L were put in the wells. Then 20  $\mu$ L of bacterial culture was inoculated. Positive control contained ciprofloxacin, while negative control contained nutrient broth and microbial strains. The 96-well plate was sealed and put to incubator at 37°C for 24 h. Under aerobic condition. After incubation, free floating bacterial cells were removed by pipetting, and then washed 2 times with PBS (pH 7.1). Then microplate was left to dry at room temperature for 15 min, and the biofilm was fixed by heating for 60 min at 60°C in the oven. Then 200  $\mu$ L of crystal violet solution (.1%) was added to all wells and left in the plate for 20 min. Crystal violet solution was removed. Each well was washed with 200  $\mu$ L of PBS to remove unbound dye. Then, 200  $\mu$ L of ethanol 95% was added to the wells, and then plate left at 4°C for 30 min to solubilize the crystal violet stained biofilm mass. The absorbance of treated and control wells was reported at 630 nm using micro plate reader (Biotek USA).<sup>16</sup>

### Phase Contrast Microscopy

Morphology of bacteria attached to material surfaces was discovered by well-reputed technique of microscopy.<sup>17</sup> The microscopy was performed from the Hi-Tech laboratory, University of Agriculture, Faisalabad, Pakistan. The sterilized glass slides were used for this assay. 25  $\mu$ L of nutrient broth, 25  $\mu$ L of sample, and 5  $\mu$ L of bacterial solution were poured on to the center of glass slide. Slides were incubated at 37 C overnight. Next day, the slides were washed with phosphate buffer with a pH of 7.8. Then, they were fixed with 99% of methanol and dried. The glass slides were stained with 7% crystal violet for 10 minutes. The slides were washed with tap water to remove extra stain. Slides were left to dry and then images were taken under a phase contrast microscope.

### Quorum Sensing Inhibition

This test was performed as described by Abdullah et al. with some modifications.<sup>18</sup> The strain *Chromobacterium*

*psuedoviolaceum* was procured from Leibniz institute DSMZ-German collection of microorganism and cell cultures, in Germany. *Chromobacterium psuedoviolaceum* was grown in LB broth (Oxoid UK) by incubating it for 24 h at 26°C. Next day, it was diluted 1:1000 with LB broth to get a bacterial concentration of 10<sup>6</sup> cfu/mL. Synthetic compound samples (10 mg/mL) serially 2-fold diluted with LB into a 48-well microplate. A bacterial culture (500  $\mu$ L) (10<sup>6</sup> CFU/mL) was added to 500  $\mu$ L of already determined dilutions of samples. When samples were prepared, the plate was incubated aerobically at 26°C without shaking for 36–48 h. Diluted bacterial culture without treatment was used as control. After incubating, 750  $\mu$ L from each well (Test and control wells) was put to 1 mL Eppendorf and centrifuge (SIGMA Laborzentrifugen Model 1–14) at 8000g for 5 min to centrifuge violacein and bacterial cells. The supernatant discarded and the pellets were vortexed (Velp Scientifica Model F28228176) with 750  $\mu$ L of 100% DMSO to solubilize the violacein. The samples were centrifuged again to precipitate bacterial cells. Now to evaluate violacein, 200  $\mu$ L of supernatant-containing violacein was put into 96-well microplate and OD<sub>585</sub> was taken. To ensure QS inhibition took place without killing the *C psuedoviolaceum* by the sample's sub-MICs, the bacterial cells which were in pellet were mixed again with 750  $\mu$ L of DW (pH 7.0) and OD<sub>595</sub> was measured. The absorbance of cells treated with sub-MICs of samples was compared with non-treated cells.

### Cytotoxicity of Synthetic Compounds

Hemolytic activity of synthetic compounds was evaluated by using the protocol of Saleem et al.<sup>19</sup> In the falcon tube (15 mL), 3 mL fresh human blood was collected. Centrifuge the blood for 5 minutes at 850 x g. The supernatant was discarded, and blood cells were undergone washing with chilled PBS for 3 times each and then diluted it to 20 mL. Then mix 20  $\mu$ L of sample and 180  $\mu$ L of prepared blood cells in Eppendorf. Eppendorf were centrifuged for 5 minutes at 1310 x g after incubating at 37°C for 30 minutes. 100  $\mu$ L supernatant was taken and mixed with 900  $\mu$ L of chilled PBS. 200  $\mu$ L from this was put to 96-well plate for absorbance at 576 nm by microplate reader (BioTek USA). Phosphate buffer saline was taken as negative control and Triton X-100 was taken as positive control. The data collected were presented in percentage of hemolysis.

### Statistical Analysis

Each experiment was performed in triplicate form. All calculations were performed by using Minitab 17 statistical software. One-way analysis of variance followed by Tukey's test was applied to determine the significant and nonsignificant.<sup>19</sup>

## Results and Discussion

### Antibacterial Activity

Antimicrobial potential of synthetic compounds (18) was evaluated against 2 *Gram* negative and 2 *Gram* positive isolates of sinusitis and comparing with standard antibiotic ciprofloxacin (Quinolone) as shown in Table 2. The compounds showed higher antibacterial potential against Gram positive strains, while moderate to low against Gram negative strains. Among pyrazole derivatives (Q series) the maximum zone of inhibition was shared by Q1 (18 mm) and Q7 (20 mm) against *S. aureus* and *E. faecalis*, respectively. Whereas in M and UA series higher zone of inhibition was observed in M3 (17 mm) and UA2 (17 mm) against above Gram positive strains, respectively. The diameter of inhibition against Gram negative *Proteus mirabilis* and *Klebsiella pneumoniae* by pyrazole compounds was demonstrated by Q3 (11 mm) and Q1 (12 mm), respectively, whereas the highest susceptibility of *P. mirabilis* and *K. pneumoniae* toward diene dione derivatives (UA, M) was shared by M3 (14 mm, 13 mm) sequentially. According to studies, substitution of electron withdrawing groups in molecules is responsible for antibacterial activity. Our all pyrazole compounds have chloride, carbonyl groups which are electron withdrawing groups responsible for good antibacterial activities except Q4 and Q6 sharing poor activity against pathogenic strains while diene dione derivatives (UA, M series) also contain carbonyl group which impart antibacterial property to these compounds as well. In a study, newly

synthesized pyrazole derivative compound (61) has shown excellent activity against multidrug-resistant strains of *S. aureus*, *P. aeruginosa*, and *E. faecium*. Among all pyrazole derivatives when py11 compared with antibiotic melittin it demonstrated 2-fold, 2–4-fold, and 4-fold better antibacterial potential against resistant strains of *S. aureus*, *P. aeruginosa*, and *E. faecium* respectively.<sup>20</sup> According to another study, new synthetic compounds (Pyrazole derivatives) from (3a-h) have revealed moderate to very good bactericidal potential against all strains, namely, *E. coli*, *S. aureus*, *P. aeruginosa* and *S. pyogenes* when compared with standard antibiotic ampicillin. The author added that the cause of variable activity against both Gram positive and Gram negative bacteria was attributed to structural differences between the two. Gram negative bacteria have an outer phospholipidic membrane. This makes the cell wall impermeable to lipophilic solutes, while Gram positive bacteria were more susceptible because of only one outer peptidoglycan layer which is not an effective permeable barrier.<sup>21</sup> This is in accordance with our study that most of our derivatives contain hydrophobic methyl groups, which make these compounds probably less effective against Gram negative pathogens (Figure 1).

### Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) for *S. aureus* determined by micro-dilution method in the presence of compound Q1 and M3 was .312 mg/mL. The concentration

**Table 2.** Antibacterial Activity of Series of Synthetic Compounds Against Bacteria Isolated from Sinusitis Patients.

Sample	<i>S. aureus</i>		<i>E. Faecalis</i>		<i>P. mirabilis</i>		<i>K. pneumoniae</i>	
	ZOI (mm)	MIC (mg/mL)	ZOI (mm)	MIC (mg/mL)	ZOI (mm)	MIC(mg/mL)	ZOI (mm)	MIC (mg/mL)
Q1	18 ± 1.3 <sup>b</sup>	.312 ± 1.4 <sup>e</sup>	15 ± 1.0 <sup>e</sup>	1.25 ± 1.01 <sup>d</sup>	10 ± 0.9 <sup>d</sup>	5 ± 0.7 <sup>b</sup>	12 ± 1.3 <sup>b</sup>	2.5 ± 0.7 <sup>c</sup>
Q2	10 ± 0.2 <sup>fg</sup>	1.25 ± 1.0 <sup>d</sup>	10 ± 1.2 <sup>i</sup>	2.5 ± 1.0 <sup>c</sup>	7 ± 1.0 <sup>f</sup>	10 ± 1.0 <sup>a</sup>	10 ± 1.2 <sup>d</sup>	2.5 ± 1.0 <sup>c</sup>
Q3	13 ± 1.0 <sup>de</sup>	1.25 ± 1.0 <sup>d</sup>	14 ± 0.3 <sup>f</sup>	1.25 ± 1.5 <sup>d</sup>	11 ± 0.3 <sup>c</sup>	2.5 ± 1.0 <sup>c</sup>	10 ± 1.0 <sup>d</sup>	2.5 ± 1.0 <sup>c</sup>
Q4	—	—	5 ± 1.1 <sup>l</sup>	10 ± 1.1 <sup>a</sup>	5 ± 1.0 <sup>g</sup>	10 ± 1.0 <sup>a</sup>	7 ± 1.1 <sup>e</sup>	10 ± 1.0 <sup>a</sup>
Q5	12 ± 1.2 <sup>de</sup>	1.25 ± 1.1 <sup>d</sup>	16 ± 0.4 <sup>d</sup>	1.25 ± 1.2 <sup>d</sup>	5 ± 1.1 <sup>g</sup>	10 ± 1.0 <sup>a</sup>	7 ± 1.0 <sup>e</sup>	10 ± 0.5 <sup>a</sup>
Q6	6 ± 1.2 <sup>j</sup>	10 ± 1.2 <sup>a</sup>	6 ± 1.0 <sup>k</sup>	10 ± 1.0 <sup>a</sup>	—	—	—	—
Q7	13 ± 1.3 <sup>d</sup>	1.25 ± 0.7 <sup>c</sup>	20 ± 1.3 <sup>b</sup>	.156 ± 0.7 <sup>e</sup>	10 ± 1.4 <sup>d</sup>	10 ± 0.7 <sup>a</sup>	11 ± 1.1 <sup>c</sup>	5 ± 0.7 <sup>b</sup>
Q8	6 ± 1.0 <sup>l</sup>	10 ± 1.0 <sup>a</sup>	7 ± 0.1 <sup>i</sup>	10 ± 0.4 <sup>a</sup>	—	...	7 ± 1.0 <sup>e</sup>	5 ± 1.0 <sup>b</sup>
UA1	10 ± 1.6 <sup>fg</sup>	2.5 ± 0.1 <sup>c</sup>	12 ± 1.2 <sup>h</sup>	5 ± 0.7 <sup>b</sup>	13 ± 1.2 <sup>b</sup>	2.5 ± 0.7 <sup>c</sup>	10 ± 0.8 <sup>d</sup>	5 ± 0.7 <sup>b</sup>
UA2	7 ± 0.8 <sup>hi</sup>	10 ± 0.7 <sup>a</sup>	17 ± 0.4 <sup>c</sup>	5 ± 1.4 <sup>b</sup>	10 ± 1.3 <sup>d</sup>	2.5 ± 0.8 <sup>c</sup>	11 ± 0.6 <sup>c</sup>	2.5 ± 0.8 <sup>c</sup>
UA3	16 ± 0.9 <sup>c</sup>	1.25 ± 0.7 <sup>d</sup>	15 ± 0.8 <sup>e</sup>	10 ± 1.4 <sup>a</sup>	9 ± 1.1 <sup>e</sup>	2.5 ± 0.7 <sup>c</sup>	12 ± 1.3 <sup>b</sup>	2.5 ± 0.7 <sup>c</sup>
UA4	—	—	—	—	5 ± 1.0 <sup>g</sup>	10 ± 1.0 <sup>a</sup>	5 ± 1.0 <sup>f</sup>	10 ± 1.0 <sup>a</sup>
UA5	6 ± 0.7 <sup>ji</sup>	10 ± 1.1 <sup>a</sup>	5 ± 1.0 <sup>a</sup>	10 ± 1.0 <sup>a</sup>	—	—	—	—
M1	9 ± 1.0 <sup>g</sup>	5 ± 1.4 <sup>b</sup>	—	—	—	—	—	—
M2	12 ± 1.1 <sup>e</sup>	1.25 ± 1.0 <sup>d</sup>	10 ± 1.1 <sup>c</sup>	2.5 ± 1.4 <sup>i</sup>	—	—	7 ± 1.0 <sup>e</sup>	10 ± 1.0 <sup>a</sup>
M3	17 ± 1.2 <sup>c</sup>	.312 ± 0.8 <sup>e</sup>	13 ± 0.9 <sup>g</sup>	2.5 ± 0.8 <sup>c</sup>	10 ± 1.7 <sup>d</sup>	1.25 ± 0.7 <sup>d</sup>	11 ± 2.0 <sup>c</sup>	2.5 ± 0.7 <sup>c</sup>
M4	8 ± 1.0 <sup>h</sup>	5 ± 1.0 <sup>b</sup>	10 ± 1.3 <sup>i</sup>	2.5 ± 0.5 <sup>c</sup>	10 ± 1.1 <sup>d</sup>	2.5 ± 1.0 <sup>c</sup>	5 ± 1.0 <sup>f</sup>	10 ± 1.1 <sup>a</sup>
M5	10 ± 1.2 <sup>f</sup>	5 ± 1.1 <sup>b</sup>	—	—	—	—	7 ± 1.0 <sup>e</sup>	5 ± 1.0 <sup>b</sup>
Ciprofloxacin	28 ± 0.5 <sup>a</sup>	.039 ± 0.2 <sup>e</sup>	26 ± 0.4 <sup>a</sup>	.078 ± 0.4 <sup>e</sup>	30 ± 1.2 <sup>a</sup>	.019 ± 0.4 <sup>e</sup>	28 ± 1.4 <sup>a</sup>	.019 ± 0.2 <sup>d</sup>

*S. aureus* = *Staphylococcus aureus*, *E. faecalis* = *Enterococcus faecalis*, *P. mirabilis* = *Proteus mirabilis*, *K. pneumoniae* = *Klebsiella pneumoniae*. (–) = No activity. MIC Results are shown as n = 3, mean ± standard deviation. Furthermore, means carrying different superscripted alphabet (a-i) exhibit significant difference among means at (P< .05) with 95% confidence interval.

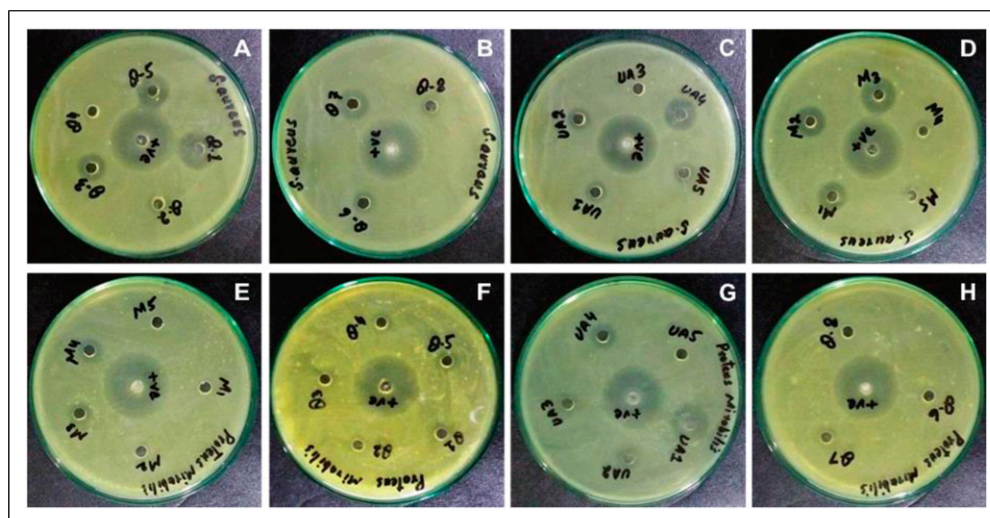


required to inhibit growth of *E. faecalis* was .156 mg/mL, documented by Q7. The MIC was as low as 1.25 mg/mL toward *P mirabilis*. The MIC was as low as 1.25 mg/mL toward *P mirabilis* shared by M3. *K. pneumoniae* growth was inhibited at the lowest concentration of 2.5 mg/mL shared by Q1, Q2, Q3, UA2, UA3, and M3 as shown in Table 2 (Figure 2).

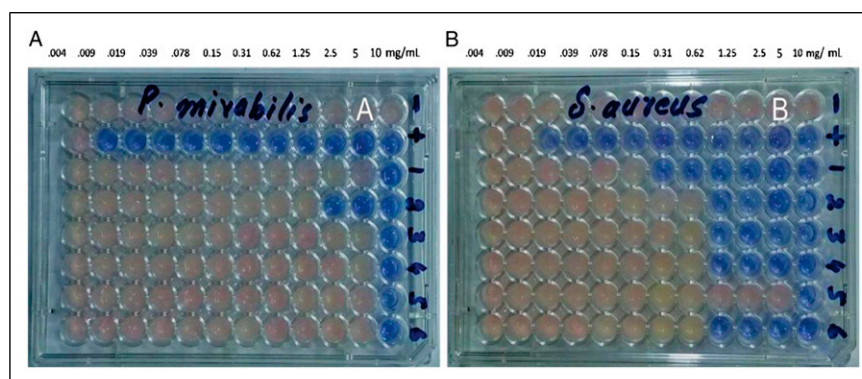
### Biofilm Inhibition of Synthetic Compounds

Biofilms consist of extra polymeric substances (EPS) occupied by microbial communities. These have the ability to adhere to abiotic and biotic surfaces.<sup>21</sup> According to National Institute of Health (NIH), about 65–80% bacterial and chronic infections are due to biofilm formation.<sup>22</sup> In 2017, World Health Organization (WHO) has issued a list of priority pathogens in which majority of pathogens belong to Gram negative class. This is because of structural differences in the cell wall of Gram negative bacteria as compared to Gram

positive bacteria.<sup>23</sup> Synthetic compounds were evaluated for their biofilm inhibitory potential against 4 pathogenic strains of sinusitis against reference drug ciprofloxacin, as shown in Table 3. Although the measured anti-biofilm potential was from whole molecule but limited structural activity relationship (SAR) was also observed. The compounds like Q (65.55), Q2 (40.43), Q3 (47.51), Q5 (45.61), Q7 (49.72), UA1 (43.45), UA3 (57.61), M2 (53.46) and M3 (69.52) had shown appreciable percentage biofilm inhibition potential then rest of the compounds against *S aureus* and *E. faecalis*, respectively. As mentioned earlier, compounds have given moderate to poor percentage biofilm hydrolysis against Gram negative pathogens such as *P mirabilis* and *K. pneumoniae* Q1 (33.41), Q2 (24.36), Q3 (29, 31), Q5 (10, 21), Q7 (39, 40), UA1 (42, 46), UA3 (35, 45), M2 (5, 25), and M3 (45, 34), respectively. The presence of methyl and carbonyl or chloride groups are common in above compounds, which enhances their anti-biofilm activity.



**Figure 1.** Representative plates (A–D) and (E–H) present antibacterial activity of synthetic compounds against *Staphylococcus aureus* and *Proteus mirabilis*, respectively. Ciprofloxacin is use as a standard in the center.



**Figure 2.** (A) Representative plate of minimum inhibitory concentration of synthetic compounds against *Proteus mirabilis*. (–) = Negative, (+) = Ciprofloxacin, 1. Q1, 2. Q2, 3. Q3, 4. Q4, 5. Q5, 6. Q6. (B) Representative plate of minimum inhibitory concentration of synthetic compounds against *Staphylococcus aureus*. (–) = Negative, (+) = Ciprofloxacin 1. Q1, 2. Q2, 3. Q3, 4. Q5, 5. Q6, 6. Q7.

A study reported the synthesis and antibacterial potential of 31 coumarin-substituted pyrazole derivatives. Out of 31 compounds, some have shown remarkable activity against MRSA strain with MIC around 3.125 µg/mL. These molecules were also effective at inhibition and destruction of biofilms of MRSA.<sup>24</sup> In another study, synthesized 2 pyrazole derivatives and evaluated their antimicrobial and anti-biofilm potential against 4 *Salmonella spp.* Both compounds inhibited biofilm formation with reduction up to 5.2 log<sub>10</sub>.<sup>25</sup> The author further stated that fluorine and methyl group confer lipophilicity which in turn increases cell permeability. The presence of fluorine (Halogens) results in breakdown of extracellular matrix, thus allows penetration of compounds into biofilm. These results are in agreement with our study which also

shows presence of hydrophobic halogenated, methyl and carbonyl groups.<sup>26</sup>

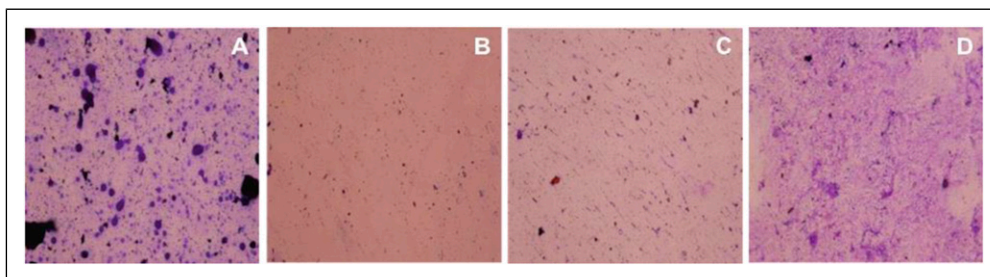
### Phase Contrast Microscopy (Qualitative Assay)

Phase contrast microscopy was performed on glass slides, which is a qualitative analysis of biofilm inhibition as shown in Figure 3. Slide (A) negative control showing heavy growth and compact area of biofilm of *Staphylococcus aureus*, Slide (B) positive control (ciprofloxacin) revealing more clear area showing little biofilm formation. Sample Q1(C) has shown significant biofilm hydrolysis on the glass slide showing more clear areas with minimum growth in comparable to standard slide (B) and UA4 compound showing more compact color areas of biofilm, confirming less biofilm inhibition by this

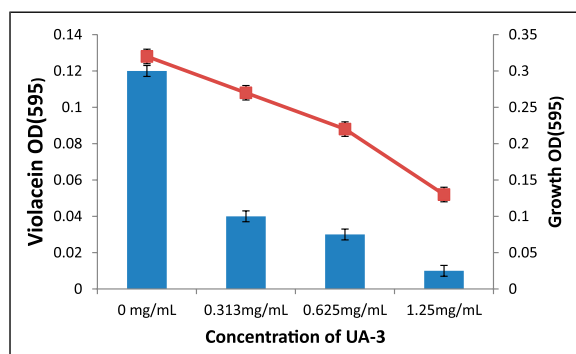
**Table 3.** Biofilm Inhibition (%) of Selected Sinusitis Isolates by Series of Synthetic Compounds.

Strain	<i>Staphylococcus aureus</i>	<i>Enterococcus faecalis</i>	<i>Proteus mirabilis</i>	<i>Klebsiella pneumoniae</i>
Sample no.	Biofilm inhibition (%) ± S. D	Biofilm inhibition (%) ± S. D	Biofilm inhibition (%) ± S. D	Biofilm inhibition (%) ± S. D
Q1	65 ± 0.3 <sup>a</sup>	55 ± 0.1 <sup>d</sup>	33 ± 0.3 <sup>d</sup>	41 ± 0.1 <sup>ab</sup>
Q2	40 ± 0.3 <sup>f</sup>	43 ± 0.4 <sup>e</sup>	24 ± 1.1 <sup>e</sup>	36 ± 1.0 <sup>c</sup>
Q3	47 ± 1.1 <sup>cde</sup>	51 ± 0.3 <sup>d</sup>	29 ± 0.3 <sup>d</sup>	31 ± 1 <sup>d</sup>
Q4	5 ± 0.1 <sup>i</sup>	6 ± 0.1 <sup>g</sup>	10 ± 0.3 <sup>f</sup>	24 ± 1.2 <sup>e</sup>
Q5	45 ± 1.0 <sup>cdef</sup>	61 ± 0.1 <sup>bc</sup>	10 ± 1.0 <sup>f</sup>	21 ± 0.3 <sup>ef</sup>
Q6	12 ± 1.1 <sup>ij</sup>	10 ± 0.5 <sup>f</sup>	5 ± 0.2 <sup>g</sup>	5 ± 0.6 <sup>h</sup>
Q7	49 ± 1.2 <sup>cd</sup>	72 ± 0.8 <sup>a</sup>	39 ± 0.9 <sup>c</sup>	40 ± 0.1 <sup>bc</sup>
Q8	19 ± 0.7 <sup>h</sup>	14 ± 1.2 <sup>f</sup>	5 ± 0.5 <sup>g</sup>	45 ± 0.2 <sup>ab</sup>
UA1	43 ± 0.4 <sup>ef</sup>	45 ± 0.4 <sup>e</sup>	42 ± 0.3 <sup>ab</sup>	46 ± 0.3 <sup>a</sup>
UA2	25 ± 0.8 <sup>g</sup>	67 ± 0.4 <sup>b</sup>	31 ± 0.6 <sup>d</sup>	43 ± 0.8 <sup>ab</sup>
UA3	57 ± 0.5 <sup>b</sup>	61 ± 0.6 <sup>c</sup>	35 ± 0.9 <sup>c</sup>	45 ± 0.2 <sup>a</sup>
UA4	5 ± 0.4 <sup>j</sup>	4 ± 0.3 <sup>g</sup>	10 ± 0.4 <sup>f</sup>	16 ± 0.3 <sup>fg</sup>
UA5	17 ± 0.6 <sup>hi</sup>	11 ± 0.6 <sup>f</sup>	5 ± 0.4 <sup>g</sup>	5 ± 0.4 <sup>h</sup>
M1	39 ± 0.5 <sup>def</sup>	5 ± 1.1 <sup>g</sup>	5 ± 0.5 <sup>g</sup>	5 ± 0.5 <sup>h</sup>
M2	53 ± 0.1 <sup>bc</sup>	46 ± 1.5 <sup>e</sup>	5 ± 0.6 <sup>g</sup>	25 ± 0.9 <sup>e</sup>
M3	69 ± 0.2 <sup>a</sup>	52 ± 1.6 <sup>d</sup>	45 ± 0.3 <sup>a</sup>	34 ± 0.1 <sup>d</sup>
M4	29 ± 0.7 <sup>g</sup>	45 ± 1.2 <sup>e</sup>	39 ± 1.1 <sup>bc</sup>	15 ± 0.2 <sup>g</sup>
M5	41 ± 0.9 <sup>def</sup>	5 ± 1.1 <sup>g</sup>	5 ± 1.3 <sup>g</sup>	20 ± 0.4 <sup>e</sup>
Ciprofloxacin	84 ± 0.9	89 ± 1.1	94 ± 1.3	90 ± 0.4

*S aureus* = *Staphylococcus aureus*, *E faecalis* = *Enterococcus faecalis*, *P mirabilis* = *Proteus mirabilis*, *K. pneumoniae* = *Klebsiella pneumoniae*. Results are shown as n = 3, mean ± standard deviation. Furthermore, means carrying different superscripted alphabet (a-j) exhibit significant difference among means at (P < .05) with 95% confidence interval.



**Figure 3.** Representative slides of phase contrast microscopy of compounds against *Staph aureus*. (A) Negative control, (B) positive control (Ciprofloxacin) (C) Q1 (D) UA4.



**Figure 4.** Production of violacein by *C. pseudoviolaceum* in comparison with bacterial growth in presence of Synthetic compound UA-3.

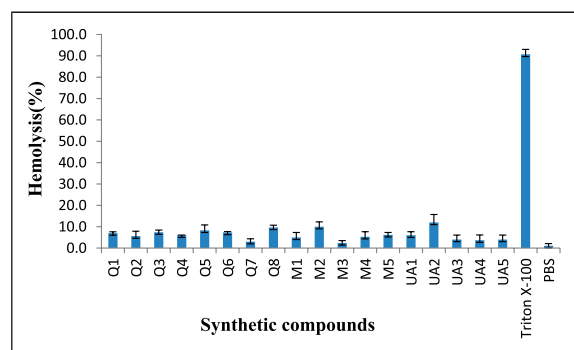
compound. These results were in accordance with the quantitative biofilm inhibition assay.

### Quorum Sensing Inhibition of Synthetic Compounds by Using *Chromobacterium pseudoviolaceum*

The effect of synthetic compounds on violacein production was studied as shown in Figure 4. *C. pseudoviolaceum* was used as a reporter strain to evaluate quorum sensing inhibition in Gram negative bacteria. In the present research work, *P. mirabilis* and *K. pneumoniae* were Gram negative pathogen isolated from sinusitis patients. The compound will be considered quorum sensing inhibitor when *C. pseudoviolaceum* strain was unable to form violet pigment (violacein) without effecting the growth of bacteria and if violacein is formed then no quorum sensing inhibition occur. The outcome displayed quorum sensing inhibition with value which was lesser than minimum inhibitory concentration of that compound. The tested concentration of synthetic compound (UA-3) .313 mg/mL inhibited violacein production significantly (OD 0.04) with very little effect on growth of *C. pseudoviolaceum* (OD 0.28) while compared to positive control having violacein production (OD 0.12) and bacterial count of (OD 0.33). However, 1.25 mg/mL showed more than 90% QS inhibition but with bacterial growth inhibition compared with positive control (bacterial cells without compounds). QS inhibition is important to reduce the production of virulence factors released by pathogenic microorganism.

### Cytotoxicity Studies of Synthetic Compounds

When the drug is going to develop, safety of the product is a major concern. Many compounds have shown toxicity issues in terms of hemolysis, which results in lysis of RBCs. Free hemoglobin cause problems for important organs like liver, heart and kidneys. Three series of synthetic compounds had given different hemolytic activities in the range of 2–12%. Most of the compounds were nontoxic toward red blood cells.



**Figure 5.** Percentage Hemolytic Activity of Synthetic Compounds Against RBC Triton X-100 (positive control); PBS (negative control).

Except two, compounds like M2 and UA2 have demonstrated some degree of cytotoxicity of 10 and 12%, respectively. Our study has been endorsed by another study who had made derivatives of pyrazole compounds by Gewald synthesis. They documented non-toxicity of 13 pyrazole derivative compounds whose hemolysis was ranging from 3.6 to 20.1% (Figure 5).<sup>27</sup>

### Conclusion

It was concluded from the current study that among 18 compounds Q3A, Q3B, Q5A, Q7A, Q9A, UA1, UA4, M2, and M5 exhibited promising antibacterial and anti-biofilm potential against Gram positive bacteria with better safety profile in terms of cytotoxicity. Second, compound UA3 had demonstrated reasonable quorum sensing inhibition against *Chromobacterium pseudoviolaceum*. Hence, the biofilm formation of Gram negative strains of *P. mirabilis* and *K. pneumoniae* causing chronic sinusitis can be inhibited by inhibition of quorum sensing in them. Further in vitro studies for efficacy and safety are recommended for these compounds.

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