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Novel curcumin analogs act as antagonists to control nosocomial infection causing *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa has become the important cause of hospital acquired nosocomial infection which is one of the leading cause of death in today's world. Here, we synthesized structurally similar analogs of curcumin (G0-G4) as antagonist to the growth and virulence of *P. aeruginosa.* The structural skeleton of analogs comprises of central bridging ligands of cyclic ketones which are flanked by side chain made up of N-Boc protected piperidinylthiazole moiety. The rationale of selecting piperidinylthiazole based pharmacophore is attributed to its therapeutic potential and aromaticity similar to curcumin. Based on the structure-activity comparison, the growth inhibitory properties of test compounds are mainly driven by side-chain pharmacophore of N-Boc protected piperidinylthiazole moiety which could be used as lead for further studies.

1. Introduction

P. aeruginosa is a Gram-negative motile facultative anaerobic opportunistic bacterium which causes nosocomial infections in hospital admitted patients. It can cause diseases in animals, birds and plant insects, nematodes (Mavrodi et al., 2001). *P. aeruginosa* can colonize critical body parts such as skin, hair, urinary tract, kidney and can easily survive in hospital environments. Colonization of the medical equipment such as catheters can cause acute and chronic diseases in immuno-compromised patients (Baltch and Smith, 1994; Sankar Ganesh et al., 2018). The pathogenesis of *P. aeruginosa* strain is attributed to its virulence and capacity of producing pyocyanin, a toxic compound known to cause lung tissue damage (Smirnov and Kiprianova, 1990). It is also involved in pathogenic biofilm formation by secreting a polymeric substance known as extracellular polysaccharide (EPS) which helps in attaching the bacterial cells together. Biofilm is composed of proteins, DNAs, polysaccharides, lipid and some of the macromolecules (Vu et al., 2009). EPS is essential in the formation of biofilm matrix and provides a barrier to the entry of antimicrobial agents (Watnick and Kolter, 2000). Moreover, *P.aeruginosa* is the most widely studied causative organism of nosocomial and chronic infections and is considered a prototype of antimicrobial resistance development (Cabot et al., 2016).Figure Scheme 1

Hitherto, curcumin based inhibitors have been studied for their antibacterial activity against *P. aeruginosa* (Thimmaraju et al., 2008). Curcumin is used as medicine in Ayurveda for centuries and its analogs have shown significant therapeutic potential as antibacterial, antifungal, anticancer and immune-modulating agents (Alok Vyas et al., 2013). Their wide array of biological activity and non-toxic nature has attracted clinicians world-wide to demonstrate their druggable properties *via* clinical trials (Preetha et al., 2007; Saroj and Hemalatha, 2015). Curcumin is also used for traditional wound treatment due to effective inhibition of bacterial biofilm of *P. aeruginosa* (Krausz et al., 2015). The basic skeleton of curcumin comprises of central bridging fragment of 1, 3-keto-enol which is part of 1, 6-heptadiene moiety. This bridging fragment is co-planer with side-chains made up of furoloyl moieties. The mechanism of action is imparted *via* strong H-bonding and hydrophobic interactions of these fragments with protein receptors of biological targets such as cytokines and adhesive molecules (Srinivasan and Rajasekaran, 2016). However, the introduction of curcumin based drugs in the market is mainly limited by their weak oral bioavailability. Therefore there is upsurge of finding the novel analogs of curcumin having enhanced bio-availability and improved bio-activity.

In order to establish the structure-activity relation, it is necessary to design (Venkatasubramanian et al., 2019) and synthesize heterogeneous side-chains or bridging groups alongside the pharmacophore. Therefore, we designed and synthesized compounds G0-G4, with the aim of identifying the novel curcumin analogs having inhibitory effects on gram negative *P. aeruginosa.* Our design strategy was structurally

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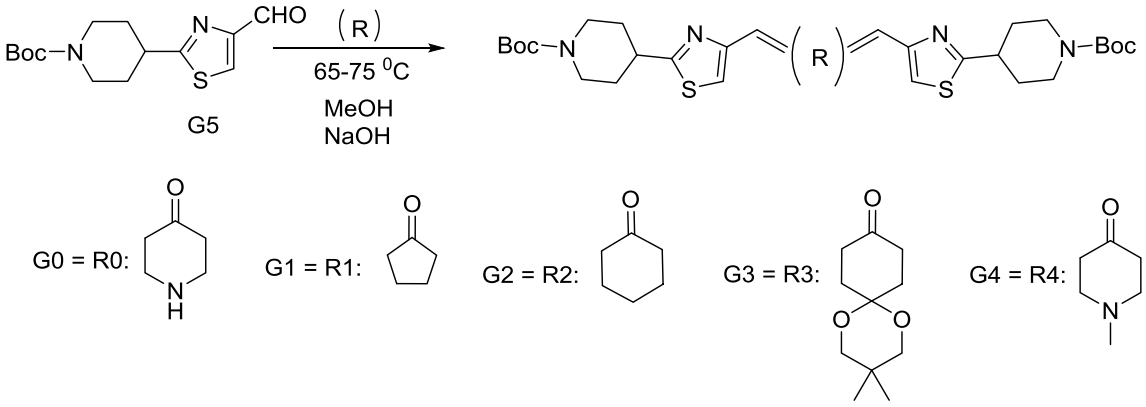

Scheme 1. Synthesis of Curcumin Analogs (Sathish et al., 2018).

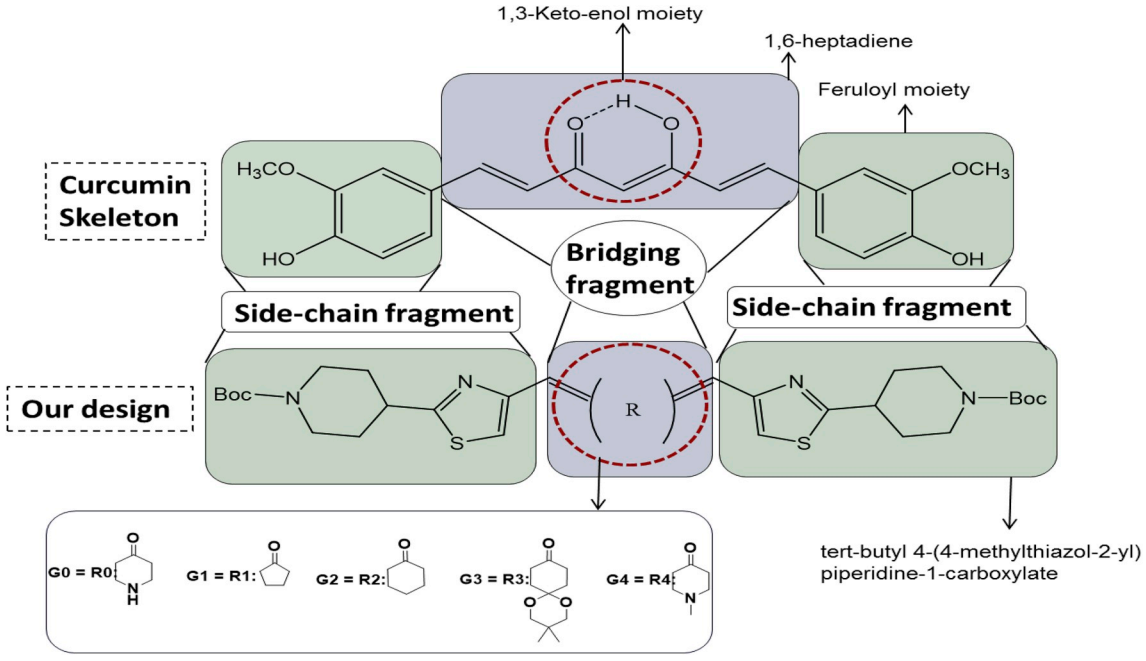

Fig. 1. Design strategy of (G0-G4): comparison with curcumin skeleton.

similar to curcumin with the side chain comprising of N-Boc protected piperidinylthiazole moiety (G5) whereas the bridging ligands were heterocyclic ketones such as cyclopentanone, cyclohexanone, 3,3-dimethyl-1,5-dioxaspiro [5.5] undecan-9-one and 1-methylpiperidin-4-one with flanking methylene groups (Fig. 1).

In this series, we used varied types of bridging fragments having low to high H-bonding propensity similar to curcumin. We envisioned that side chain of N-Boc protected piperidinylthiazole moiety would additionally enhance noncovalent association with the receptors and provide necessary steric support similar to curcumin. These compounds were evaluated for their antibacterial effect on an ATCC strain of *P. aeruginosa*. This study was further extended to determine the effect of these five compounds (G0 to G4) on virulence activity of *P. aeruginosa* such as biofilm formation, EPS and pyocyanin production. The effect on the swarming motility of *P. aeruginosa* was also evaluated.

2. Materials and methods

2.1. Materials

The chemicals cyclohexanone, cyclopentanone, N-methyl-4-piperidone, 4-piperidone and 3, 3-dimethyl-1, 5-dioxaspiro [5.5] undecan-9-one were purchased from (Sigma Aldrich). The starting material G5 was purchased from (Spincotech private limited), Chennai, India. *P. aeruginosa* ATCC 27853 was provided from the departmental stock at School of Life Sciences, BSACIST. Commercially available ethanol, crystal violet, glacial acetic acid, and sulfuric acid, Mueller Hinton broth/agar, tryptophan broth and brain heart infusion broth were purchased from (Hi-Media) Mumbai, India. Dimethylsulfoxide (DMSO), glycerol, chloroform, sodium hydroxide, hydrochloric acid and phenol were purchased (MERK) Mumbai, India.

2.2. Experimental details

Reagent grade solvents and chemicals were used as such. Bruker 400 MHz spectrometer was used to record Proton (^{1}H) and Carbon (^{13}C) NMR and TMS was used as an internal standard.

2.3. General procedure for the synthesis of curcumin analogs

To a solution of Piperidinylthiazole aldehyde G5 (2.eq) and cyclic ketone (1eq) in 10 ml of ethanol, sodium hydroxide (1eq) was added under nitrogen atmosphere and the mixture was stirred at 65 °C for 20 min, and cool to room temperature and stirred at RT for 2 h.

Reaction completion was determined by TLC, the reaction mixture was filtered and washed with 5 ml of cooled ethanol and dried at 65–70 °C for 4 h (Jayaprakash et al., 2016a and 2016b).

di-*tert*-butyl 4, 4'-(((1E,1′E)-(4-oxopiperidine-3, 5-diylidene) bis (methanylylidene)) bis (thiazole-4, 2-diyl)) bis (piperdine-1-carboxylate) G0, yield,80%, M.P = 140–150 °C. ^{1}H NMR (400 MHz,$CDCl_3$): δ = 7.60 (s,2H); 7.43 (s,2H); 4.18–4.12 (m,8H); 3.20–3.15 (m,2H); 2.55 (m,4H,); 2.14–2.12 &1.82–1.74 (m,8H); 1.49–1.47 (m,18H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 188.2, 174.3, 152.0135.3, 126.8, 126.4, 123.4, 79.7, 57.2, 45.9, 40.5, 28.5 ppm.

di-*tert*-butyl 4, 4'-(((1E,1′E)-(2-oxocyclopentane-1, 3-diylidene) bis (methanylylidene)) bis (thiazole-4, 2-diyl) bis (piperidine-1-carboxylate) G1. yield,90%, M.P = 140–150 °C FTIR *V*max (KBr): 3114,2973-2866, 1668, 1615 & 1414 cm^{-1}. ^{1}H NMR (400 MHz,$CDCl_3$): δ = 7.52 (s,2H),7.44 (s,2H), 4.18, 3.23–3.14 (br, m 8H); 2.17–1.68(m, 8H); 2.98–2.72 (m,2H); 1.83–1.649 (m,4H); 1.48 (S,18H). ^{13}C NMR (100 MHz, $CDCl_3$: δ = 197.1, 178.3, 174.2, 155.1, 155.0, 152.7, 139.2, 124.7, 122.3, 79.7, 40.5, 34.1, 28.4, 26.6 ppm. LCMS calculated for $C_{33}H_{44}N_4O_5S_2$ $(M+H)^+$ 642, found for 641.28.

di-*tert*-butyl 4, 4'-(((1E, 1′E)-(2-oxocyclohexane-1, 3-diylidene) bis (methanylylidene)) bis (thiazole-4,2-diyl) bis (piperidine-1-carboxylate) G2. yield, 90%, M.P-195-200 °C, FTIR *V* max (KBr): 3088, 2938-2855, 1681,1608,1411cm^{-1}. ^{1}H NMR (400 MHz,$CDCl_3$): δ = 7.67(S,2H); 7.39 (S,2H); 4.18, 3.24–3.15 (br,m,8H); 2.14–2.12 (m,4H); 1.87–1.80 (m,4H); 2.95–2.90 (m,2H); 1.79–1.63 (m,6H); 1.48 (S,18H). ^{13}C NMR (100 MHz,$CDCl_3$): δ = 190.7, 173.9,152.5, 154.7, 136.6,128.0, 122.3,79.7, 40.6,32.2,28.4, 22.1ppm.LCMS calculated for $C_{33}H_{46}N_4O_5S_2$ $(M+H)^+$655, found for 654.28.

di-*tert*-butyl 4, 4'-(((1E, 1′E)-(3, 3-dimethyl-9-oxo-1,5-dioxaspiro [5.5] undecane-8, 10-diylidene) bis (methanylyidene)) bis (thiazole-4,2-diyl)) bis (piperdine-1-carboxylate) G3, yield is 85%, M.P = 140–150 °C; FTIR *V*max(KBr):3088,2937,2855,1740,1681,1608 cm^{-1}. ^{1}H NMR (400 MHz,$CDCl_3$): δ = 7.46(s,2H); 7.73(s,2H); 3.76(m,4H); 3.58–4.50(br,4H); 3.22–3.19(m,4H); 2.96 (m,2H); 2.15-2.19,1.67-1.89, (m,4H); 1.67–1.86 (m,8H); 1.55–1.32(m,18H); 0.99(m,6H). ^{13}C NMR (100 MHz $CDCl_3$): δ = 189.3, 174.2, 154.7, 152.3, 132.2, 129.4, 123.1, 96.6, 79.7, 40.6, 35.2, 32.2, 30.0, 28.4, 22.7 ppm. LCMS calculated for $C_{39}H_{54}N_4O_7S_2$ $(M+H)^+$755, found for 754.34.

di-*tert*-butyl 4,4'-(((1E,1′E)-(1-methyl-4-oxopiperidine-3, 5-diylidene) bis (methanylyidene)) bis (thiazole-4,2-diyl)) bis (piperdine-1-carboxylate) G4, yield,90%, M.P = 150–160 °C; ^{1}H NMR (400 MHz,$CDCl_3$): δ = 7.60 (s,2H); 7.42 (s,2H); 4.49–4.18(m,8H); 3.20–2.70(m,10H); 2.44–1.77(m,16H); 1.48 (m,18H) ppm. ^{13}C NMR (100 MHz,$CDCl_3$): δ = 189.0,174.4,154.7,152.1,136.1,135.3,126.3,123.3, 79.7, 48.1,40.6,40.5, 28.5 ppm.

2.3.1. tert-butyl 4-(5-formylthiazol-2-yl)piperidine-1-carboxylate, G5

Purity 85.0%, ^{1}H NMR (400 Mhz,DMSO-d_6): δ = 9.86 (s,1H,) 8.62 (s,1H),4.04–3.97, 2.88 (m,4H), 3.35–3.23 (m,1H),2.04–2.01, 1.60–1.50(m,4H),1.38,(s,9H) ppm.

2.3.2. MIC and MBC of compounds

The minimum inhibitory concentration (MIC) or the minimum concentration of compounds that inhibited the bacterial growth was performed through micro broth dilution technique following CLSI guidelines (CLSI, 2016). MBC or the lowest concentration of compound that completely reduced the growth of bacteria after18-24 h was calculated by plating all MIC dilutions lacking observable turbidity. 10 µl aliquots from MIC dilutions were plated on MHA plates and incubated at 37 °C for 24 h (Saroj et al., 2018).

2.3.3. Growth curves of P. aeruginosa treated with compounds

ATCC strain of *P. aeruginosa* was grown over night at 37 °C tryptophan liquid medium until it reached its log phase. Later, the culture was transferred into a fresh tryptophan broth medium at an initial OD of 0.1. *P. aeruginosa* was treated with all the six compounds at their MIC concentration. Growth rate and the concentration of *P. aeruginosa* were spectrophotometrically recorded at 600 nm and at different time intervals (0–24 h). The results obtained were graphically plotted (Maiti et al., 2014).

2.3.4. Antimicrobial activity through well plate method

Antimicrobial activity of all the six compounds was evaluated (Umar at.al., 2019; Tahira et al., 2018) against an ATCC strain of *P. aeruginosa* strain by agar diffusion test following CLSI guidelines (CLSI, 2016). 5 mg of all the six compounds was dissolved in 1 ml of 50% dimethyl sulfoxide (DMSO). 6 mm wells were cut and 100 µl of compound was loaded into each well. 50% DMSO was used as a control and plates were incubated for 18–24 h at 37 °C. The inhibition zone was determined by measuring the diameter of the clear zone around each well.

2.3.5. Biofilm inhibition assay

The effect of compounds on biofilm formation by an ATCC (27853) strain of *P. aeruginosa* was perceived through the modified methods (Moskowitz et al., 2004). Overnight *P. aeruginosa* culture was diluted in fresh Brain heart infusion broth medium (1:100) in a tissue culture plate which contained the two-fold reducing concentrations of compounds (0.83–0.21) mcg/ml. Well with strain and no compound served as a positive control, while the well with only broth served as negative control. All the treated cultures were incubated at temperature of 37 °C for twenty four hours. The medium was discarded after proper incubation and double distilled water was used to clean the tubes followed by the addition of 0.1% crystal violet to all the wells. After 15 min of standing, the additional dye was rinsed with PBS. The emergence of biofilm was confirmed by the formation of a pinkish ring in the wells. The rings were washed with 30% acetic acid and 125 µl of the suspension was spectrophotometrically read at 520 nm.

2.3.6. Swimming and swarming assay

Swimming: Tryptone broth [10 g/liter; tryptone (Hi-Media); 5 g/liter NaCl] that contains 0.3% (w/v) agarose was used as culture media for assay. Inoculated the swim plates with bacteria which is from overnight cultured in LB medium (1.5%, w/v) plates at 37 °C with a sterile toothpick. To prevent dehydration the plates were covered with aluminum foil and incubated at 30 °C for twenty-four hours.

Swarming: Culture media used for assay consisted of 0.5% agar with 8 g/liter Nutrient broth (Hi-Media), to which 5 g/liter glucose was added. Before pouring media MIC/ml of the compound was added. Before use swarm plates were dried at room temperature overnight. When cells were inoculated onto swarm plates from swim agar (0.3%) plates followed by incubation at 30 °C, swarming efficiency was improved (Rashid and Kornberg, 2000). The swarming colony was measured using Hi-Media, zone scale and zone diameter was compared with control.

2.3.7. Pyocyanin quantification

The pyocyanin assay is based on the absorbance of pyocyanin at 520 nm in acidic solution. A 3-ml sample of culture grown in glass tube for pyocyanin production was extracted with 0.6 ml of chloroform and then re-extracted with 0.2 ml of 0.2 N-hydrochloric acids to give a pink to deep red solution. The percentage of growth of the treated strains was compared with the untreated control strains by measuring the optical density at 520 nm. Concentrations, expressed as strains of *P. aeruginosa* produced per milliliter of culture supernatant were determined by multiplying the optical density value at 520 nm by 17.072 (Kurachi, 1958).

2.3.8. EPS estimation

Quantification of EPS and extraction were performed using the method as described (Ganesh and Vittal, 2015). The test organism was grown in tryptophan broth with Compounds (G0, G1, G2, G3, G4 and

Table 1
MIC and MBC of six different compounds against an ATCC strain of *P. aeruginosa*.

MIC and MBC determination mg/ml											
G0		G1		G2		G3		G4		G5	
MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
0.41	0.82	0.41	0.82	0.41	0.82	0.41	0.82	0.43	0.86	0.43	0.86

G5) at their MIC. The untreated *P. aeruginosa* served as a control. Experiment was conducted as reported by (Saroj et al., 2019a and 2019b).

3. Results

3.1. MIC and MBC determination

The primary step in performing the antimicrobial activity of compounds (G0, G1, G2, G3, G4, and G5) was the determination of MIC and MBC against an ATCC strain of *P. aeruginosa*. MIC and MBC were carried out in Mueller-Hinton broth medium. MIC and MBC of *P. aeruginosa* strain (ATCC 25922) were recorded and are summarized in Table 1.

Where: MIC-minimum inhibitory concentration; MBC-Minimum bactericidal concentration; ATCC-American type of culture collection.

3.2. Time-kill assay

In order to examine the effect of six different compounds on the growth of an ATCC strain of *P. aeruginosa*, spectrophotometric measurements were recorded at 600 nm at different time intervals. Out of the six compounds used, six compounds (G0-G5) were more effective than the antibiotic (ceftazidime) used. *P. aeruginosa* control strain result is presented in (Fig. 2a) showed a luxuriant growth compared to the treated strains (Fig. 2b).

3.3. Antibacterial activity of compounds through well plate method

Antimicrobial effect of compounds on an ATCC strain of *P. aeruginosa* was carried on MHA media by agar diffusion method. The result was measured through the zones of inhibition determining the efficacy of the compounds to inhibit the growth of bacteria. Zones were measured with the help of Hi-Media zone scale. The zone of inhibition together with the graphical representation is summarized in (Fig. 2c and d). From the results, it can be observed that the antibiotic (ceftazidime) was more effective than the compounds in suppressing the growth of *P. aeruginosa* but the zones of inhibition obtained for all the compounds were reasonable and it can be stated that the compounds acted as good antimicrobial agents.

3.4. Biofilm, pyocyanin and EPS inhibition

All the compounds showed an inhibitory effect on the production of biofilm, pyocyanin and EPS. The compounds (G0, G1, G2, G3, G4, and G5) inhibited the biofilm formation when compared to the control strain. Biofilm was completely inhibited at lower concentrations of compounds even lower than MIC. ATCC strain of *P. aeruginosa* was treated with two-fold dilutions of compounds G0 to G5 (0.42 mg/ml) in BHI liquid medium. The graphical representation of biofilm inhibition is presented in (Fig. 3a). The pyocyanin production was also inhibited in presence of all the compounds compared to the control (without treatment) as shown in (Fig. 3c). Biofilm formation and pyocyanin

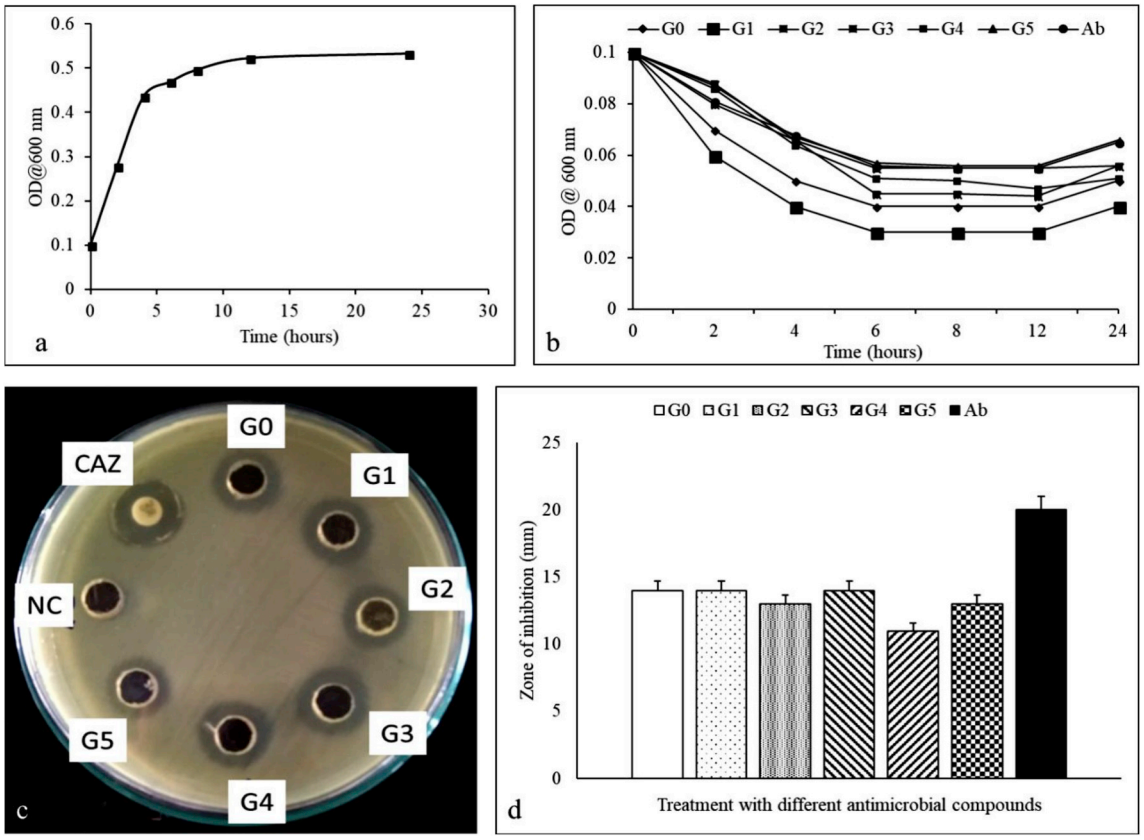

Fig. 2. (a) Time course experiment displaying the growth of an ATCC strain of *P. aeruginosa* without treatment and (b) with the treatment of compounds at different time intervals. (c) Antibacterial effect of six different compounds on an ATCC strain of *P. aeruginosa* through the well-plate method and (d) the graphical representation of the zones of inhibition obtained. The values represented are mean ± S.D.

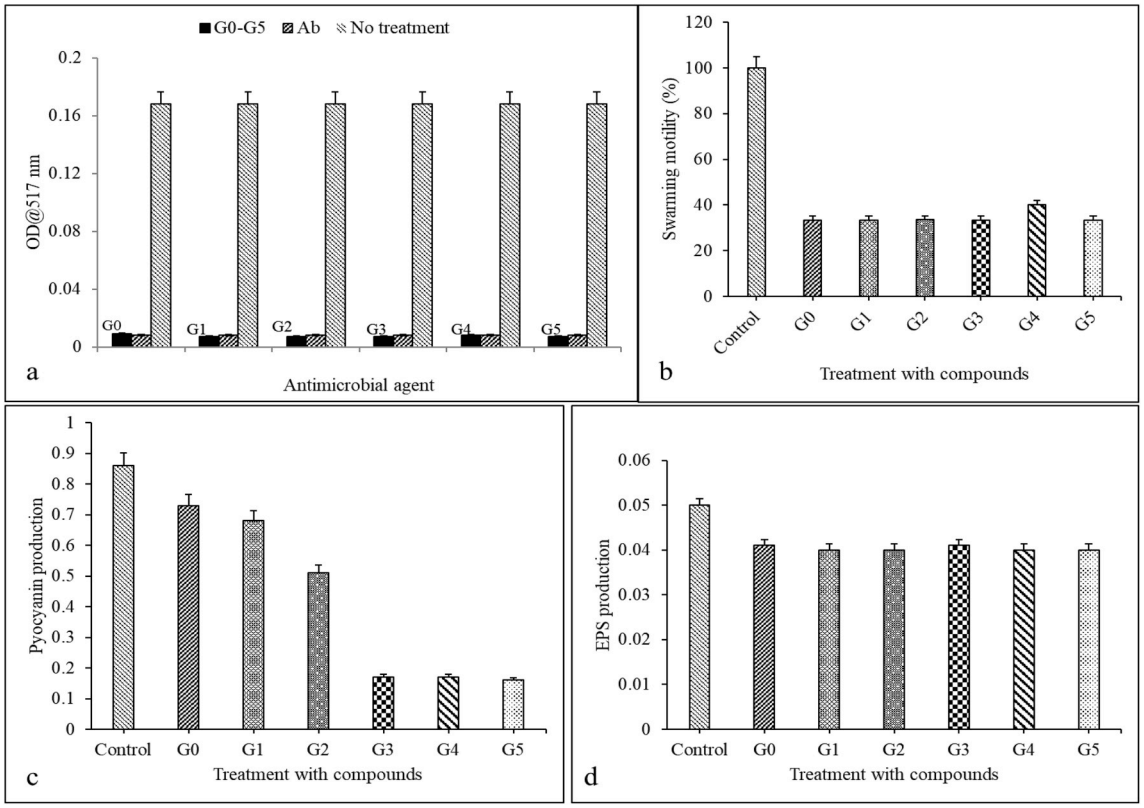

Fig. 3. (a) Biofilm inhibition in an ATCC strain of *P. aeruginosa* (b) Reduction in the motility of *P. aeruginosa* strain upon treatment with compounds (G0-G5) (c) Effect of compounds on pyocyanin production in an ATCC strain of *P. aeruginosa* (d) Reduction in EPS production was observed in the strain treated with compounds. The values represented are mean ± S.D.

production are an important part of the virulence mechanism responsible for quorum sensing in *P. aeruginosa* (Lee and Zhang, 2015). EPS production in an ATCC strain of *P. aeruginosa* is shown in (Fig. 3d). The effect of all compounds showed a similar trend in inhibiting the production of EPS. Compared to the control strain, the EPS production was reduced in *P. aeruginosa* when treated with compounds (G0-G5).

3.5. Swarming motility assay

Swarming motility in an ATCC strain of *P. aeruginosa* was observed after 48 h of incubation at 30 °C. The effect of MICs of all compounds on swarming motility was examined. The reduction in swarming motility was observed in an ATCC strain of *P. aeruginosa* treated with six different compounds (Fig. 3b).

4. Discussion

The structures of the newly synthesized compounds were characterized by spectral and analytical data. Sensitive strain of *P. aeruginosa* was used to evaluate the antimicrobial potential of five test compounds (G0-G4) analyzed through different assays such as MIC, MBC and antibacterial activity through well-plate method.

Based on the MIC data, all the compounds were found to be good antibacterial agents. The compounds G0, G1, G2 and G3 have shown higher inhibition of bacterial growth than G4. The effect of test compounds on time kill assay reveals that G1 and G0 were more active than their analogues and ceftazidime antibiotic. This observation was further corroborated by growth inhibition study by well-plate method (Ubaid et al., 2018; Ubaid and Hemalatha, 2017) The compounds G1 and G0 have shown higher zone of inhibition when compared with their analogous. However, the highest zone of inhibition of antibiotic may be attributed to its better diffusion in the solid media (Tahira et al., 2019).

The inhibition of pathogenesis of *P. aeruginosa* was evaluated by testing the effect of test compounds on the virulent properties such as pyocyanin, EPS and biofilm production through spectrophotometric measurements. The reduction in the swarming motility and the inhibitory effect on biofilm production was found to be comparable amongst all the test compounds. On the contrary, the inhibition on pyocyanin production revealed significant variation which remains unclear. Interestingly, compound G5 also showed suppressive effect on the virulence of *P. aeruginosa.* This pinpointed the common mode of action of these set of compounds.

Based on the growth inhibition and suppression of virulent activity data, it can be inferred that the nature of side-chain pharmacophore comprising N-Boc protected piperdinethiazole moiety is mainly influencing the antibacterial properties of test compounds against *P. aeruginosa*. The comparable activity of all the compounds is driven by strong steric support provided by side-chain (Srinivasan and Rajasekaran, 2016). Though the influence of bridging ligand is not significant, in order to find lead, we sought some correlation between the MIC value and time-course experiment. It was observed that the compound G1 with cyclopentanone as central moiety have shown highest bio-activity in comparison with other compounds. We propose that its low bulkiness might have attributed to its enhanced activity.

5. Conclusion

N-Boc protected piperidinylthiazole moiety may serve as important side-chain pharmacophore similar to furoloyl moiety of curcumin for antibacterial effect of this class of compounds. Further, this moiety in combination with heterocyclic bridging ligands flanked by methylene groups can be clinically explored as novel antagonist of *P. aeruginosa*. The compound G1 can be taken further as lead for its significant antibacterial effect on *P. aeruginosa*. Currently we are conducting study to

discover the mechanism of action of this class of compounds and their bio-target at molecular level.

Conflicts of interest

No declaration from authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101238.

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