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Antimicrobial activity and time kill curve study of newly synthesized dialkyl carboxylate cyclohexane derivative; A novel anti-*Pseudomonas aeruginosa* compound

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ABSTRACT. *Pseudomonas aeruginosa* causes nosocomial infections, ventilator-associated pneumonia and high morbidity and mortality in immunocompromised and cystic fibrosis patients. Development of the high level of resistance to multiple antibiotics and lack of new drugs accentuate the need of new antimicrobial substances against this opportunistic pathogen. A novel dimethyl carboxylate cyclohexane derivative was synthesized and initially screened against four Gram-positive bacteria and four Gram-negative bacteria by agar well diffusion method. Minimum inhibitory concentration (MIC) was determined against all test pathogens using resazurin dye by broth microdilution method. Effect of test compound on growth curve of *Pseudomonas aeruginosa BDU-49* was evaluated by turbidimetric method. Time kill assay was performed to assess bacteriostatic or bactericidal nature and relationship between the concentration of the test compound and the net growth rate of *Pseudomonas aeruginosa BDU-49*. Test compound exhibited better antimicrobial activity against Gram-negative bacteria. *Pseudomonas aeruginosa BDU-49* was the most susceptible test culture with MIC 62.5 µg mL^{-1}. The growth curves of *Pseudomonas aeruginosa BDU-49* demonstrated that test compound could inhibit the growth and reproduction of bacteria. Time kill assay showed that test compound is bactericidal at 2× MIC and bacteriostatic at MIC. Overall, these data indicate that test substance could act as probable novel anti-*Pseudomonas aeruginosa* compound in future.

Keywords: *Pseudomonas aeruginosa*; antimicrobial activity; microdilution method; time kill curve; minimum inhibitory concentration; drug resistanc.

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Introduction

Over the past decade, antimicrobial resistance has emerged as serious worldwide health problem for both humans as well as animals. Drug resistant bacteria are major problem in treatment and eradiation of infectious diseases (Shoaib & Ganbarov, 2019a). Intrinsic resistance (antibiotic inactivating enzyme, Over expression of efflux pumps, low outer membrane permeability, biofilm formation etc.), adaptive resistance (continuous exposure to antibiotics, over exposure to environmental stress etc.) and acquired resistance (horizontal gene transfer, mutation etc.) are proposed mechanisms of development of antimicrobial resistance (Pachori, Gothalwal, & Gandhi, 2019). Due to perpetual increasing antimicrobial resistance, worldwide drug studies are focusing development of new antimicrobial drugs.

Pseudomonas aeruginosa is a Gram-negative, motile, non-spore forming bacterium. It is found as part of normal intestinal flora. Therefore, it is an opportunistic pathogen in patients with compromised immune system (Roy et al., 2014). It causes nosocomial infections, ventilator-associated pneumonia and high morbidity and mortality in immunocompromised and cystic fifibrosis patients (Sadikot, Blackwell, Christman, & Prince, 2005; Pang, Raudonis, Glick, Lin, & Cheng, 2019). Chronic infections caused by this bacterium are difficult to treat because of its ability to form biofilm, as antibiotics can't penetrate biofilms (Ciofu & Tolker-Nielsen, 2019). Thus, it has evolved as multi drug resistance pathogen by utilizing high level of intrinsic and acquired resistance mechanisms. Over expression of efflux pump and low permeability of outer membrane of this bacterium are methods of development of innate resistance, while mechanisms of acquired resistance include = the resistance genes or mutation in genes encoding porins, penicillin-binding proteins,

chromosomal β-lactamase etc. This leads to development of multi drug resistant against various antibiotics including β-lactams, carbapenems, aminoglycosides and fluoroquinolones in *Pseudomonas aeruginosa* (Pachori et al., 2019). It has been one of the most threatning pathogens involved in antibiotic resistance which leads to wide range of infections in critically ill patients (Moore et al., 2014). This microorganism has been listed as one of three bacterial species by the World Health Organization (WHO), which requires the development of new drugs to combat infections (World Health Organization [WHO], 2017). Possible therapeutic approaches against *Pseudomonas aeruginosa* are hindered by ability of bacterium to form tolerant and resistant biofilms (Ciofu & Tolker-Nielsen, 2019). To ameliorate serious problem of antimicrobial resistance, it is need of time to formulate novel antimicrobial drugs with unique mode of action and broad therapeutic spectrum.

Functionally substituted synthetic organic compounds with higher antimicrobial profile and mode of action which is not known by resistance mechanisms of bacteria are considered as potential antimicrobial agents of future (Tsemeugne et al., 2018). Due to ever increasing antimicrobial resistance, functionally substituted cyclohexane derivatives are being widely explored as probable antimicrobial agents (Shoaib, Ismiyev, Ganbarov, Israyilova, & Umar, 2020). These cyclohexane derivatives have shown diverse biological properties i.e. antibacterial activity, antifungal activity, anticancer activity, antioxidant activity, cytotoxic activity etc. Small changes in the functional group of cyclohexane derivatives have led to accentuation of antimicrobial profile of these compounds (Flefel et al, 2014; Shoaib, Israyilova, & Ganbarov, 2019b). Functionally substituted dialkyl carboxylate cyclohexane derivatives have exhibited variable antimicrobial activity. These compounds have shown moderate to strong antibacterial activity especially against Gram-negative bacteria (Urzua, Echeverria, Rezende, & Wilkens, 2008; Shoaib et al., 2019c). Keeping in view the above mentioned facts, the aim of the current research is to report synthesis, antimicrobial activity and time kill curve study of novel functionally substituted dialkyl carboxylate cyclohexane derivative.

Material and methods

General information

All the solvents and reagents were purchased from commercial suppliers and were of analytical grade and used without further purification. The control of the reactions progress and the determination of the synthesized compounds purity were done by thin layer chromatography (TLC) on Merck silica gel plates (60 F254 aluminum sheets) which were visualized under UV light. Melting points were recorded in open capillary tubes on a Buchi B-540 apparatus and were uncorrected. Elemental analysis was performed on the Carlo Erba 1108analyzer.

NMR experiments

The NMR experiments were performed on a BRUKER FT NMR spectrometer AVANCE 300 (Bruker, Karlsruhe, Germany; 300 MHz for ^{1}H and 75 MHz for ^{13}C) with a BVT 3200 variable temperature unit in 5 mm sample tubes using Bruker Standard software (TopSpin 3.1). Chemical shifts were given in ppm (δ) and were referenced to internal tetra methyl silane (TMS). Multiplicities are declared as follow: s (singlet), d (doublet), t (triplet), q (quadruplet), m (multiplet). Coupling constants *J* are given in Hz. The experimental parameters for ^{1}H are as follows: digital resolution = 0.23 Hz, SWH = 7530 Hz, TD = 32 K, SI = 16 K, 90ºpulse-length = 10 ms, *PL*1 = 3 dB, *ns* = 1, *ds* = 0, *d*1 = 1 s and for ^{13}C as follows: digital resolution = 0.27 Hz, SWH = 17985 Hz, *TD* = 64 K, *SI* = 32 K, 90º pulse-length = 9 ms, *PL*1 = 1.5 dB, *ns* = 300, *ds* = 2, *d1* = 3 s. The NMR-grade DMSO-d_6 (99.7%, containing 0.3% H_2O) was used for the solutions of synthesized compound.

Synthesis of dimethyl-5-acetyl-1,3-dicyano-4-hydroxy-4-methyl-2,6-diphenylcyclohexane-1,3-dicarboxylate

As shown in Figure 1, cascade reaction involving multicomponent interactions of methyl 2-cyanoacetate with benzaldehyde and acetylacetone resulted in formation of new derivative; dimethyl-5-acetyl-1,3-dicyano-4-hydroxy-4-methyl-2,6-diphenylcyclohexane-1,3-dicarboxylate.

10 mL of methanol, 0.53 g (5 mmoL) of benzaldehyde and 0.99 g (10 mmoL) of methyl 2-cyanoacetate were mixed in 50 mL flask supplied with magnetic mixer. Subsequently, 2 mL of water solution of 0.1 g of NaOH was poured to the reaction medium. After 30 min., 0.5 g of acetylacetone (5 mmoL) and 0.53 g of benzaldehyde

(5 mmoL) were added to the medium. The reaction medium was kept at room temperature for 48 hours. The precipitated crystals were filtered and recrystallized from acetonitrile. Yield 71%.M.p. 157-159^{0}C. ^{1}H NMR spectrum: (DMSO-d_6, δ, ppm), 1.25 s (3H, CH_3), 2.1 s (3H, CH_3), 2.70-2.75 d (1H, CH, J = 15 Hz), 2.80-2.83 d (1H, CH, J = 9 Hz), 3.15 s (1H, CH),3.35 s (3H, OCH_3), 3.50 s (3H, OCH_3), 5.1 s (1H, OH), 7.20-7.47m (7H, 7C_{Ar}H), 7.55-7.72 m (3H, 3C_{Ar}H – Figure 2). ^{13}C NMR spectrum: (DMSO-d_6, δ, ppm), 22.15 (CH_3), 33.45 (CH_3), 40.01 (CH), 41.98 (CH), 48.9 (CH), 54.1 (C), 57.8 (C), 66.75 (OCH_3), 72.81 (OCH_3), 78.69 (C), 102.91 (CN), 105.13 (CN), 127.78 (2C_{Ar}H), 128.85 (C_{Ar}), 128.89 (C_{Ar}H), 130 (2C_{Ar}H), 132 (2C_{Ar}H), 132.85 (C_{Ar}), 133.75 (2C_{Ar}H), 134.5 (C_{Ar}H), 160.5 (COO), 162 (COO), 193.55 (CO). Found, %: C-68.65; H-5.82; N-6.03, $C_{27}H_{26}N_2O_6$. Calculated, %: C 68.34; H-5.52; N-5.90 (Figure 3).

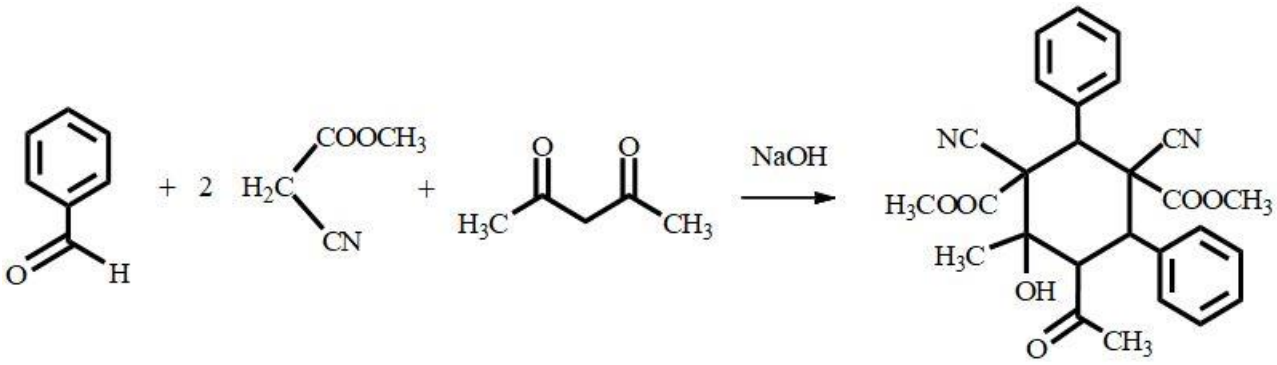

Figure 1. Cascade reaction involving multicomponent interactions.

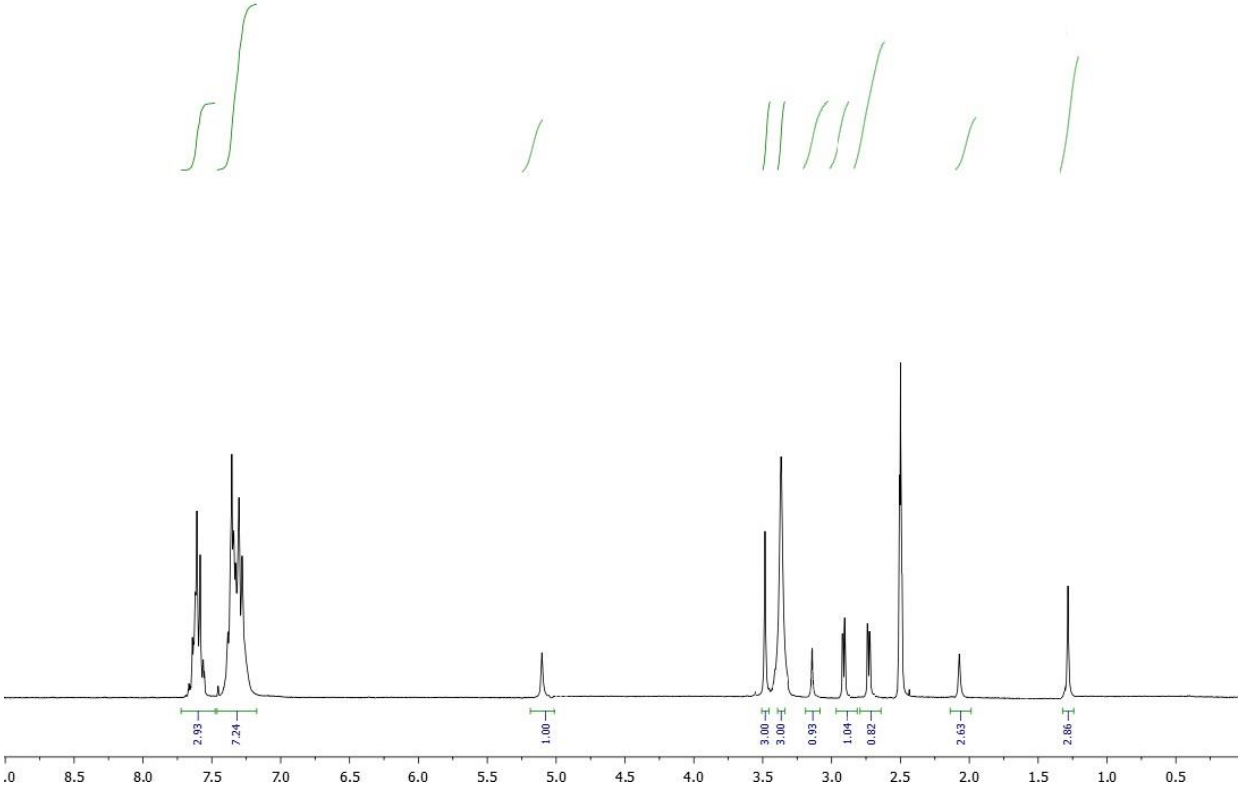

Figure 2. ^{1}H NMR spectrum of synthesized compound in DMSO-d_6 solution.

Bacterial strains and growth conditions

All the test cultures were taken from collection at department of Microbiology, Baku State University. Four Gram-negative bacteria (*Escherichia coli BDU-12, Klebsiella pneumoniae BDU-44, Acinetobacter baumannii BDU-32* and *Pseudomonas aeruginosa BDU-49*) and four Gram-positive bacteria (*Staphylococcus aureus BDU-23, Bacillus Subtilis BDU-50, Bacillus mesentericus BDU-51* and *Bacillus megaterium BDU-N20*) were used for initial screening against test compound. Mueller-Hinton ('Liofilchem') agar and broth were used to cultivate bacterial species. Freshly grown 24 hours broth cultures were used in all the experiments.

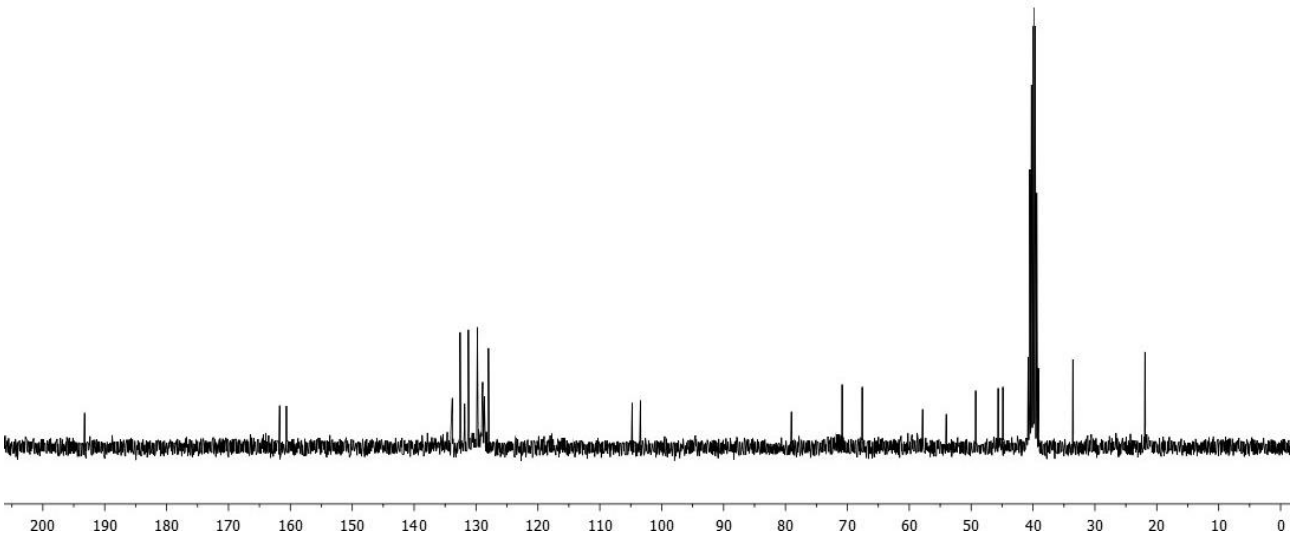

Figure 3. ^{13}C NMR spectrum of synthesized compound in DMSO-d_6 solution.

In vitro antimicrobial susceptibility testing

Agar well diffusion assay

Standard agar well diffusion assay (Balouiri, Sadiki, & Ibnsouda, 2016; Shikhaliyev et al., 2019) was used to evaluate test compounds against test cultures. Briefly, 100 µL of 0.5 McFarland bacterial suspensions were aseptically spread on Mueller-Hinton agar plates and wells with diameter 8 mm were made with the help of sterile cork borer. Due to its inert nature, dimethyl sulphoxide (DMSO) was used to dissolve test compound. Three different concentrations of test compound (0.3, 0.1 and 0.05%) were evaluated for antimicrobial activity. 150 µL of test compound was added in each well and plates were incubated at 37°C for 24 hours and average zone of inhibition was recorded. DMSO was used as control and all the experiments were conducted three times.

Determination of MIC

Minimum inhibitory concentration (MIC) values were determined by using resazurin dye by broth microdilution method (Palomino et al., 2002; Israyilova et al., 2016). In short, Mueller-Hinton broth was used to perform resazurin assay. 24 hour fresh broth culture was used and final density was adjusted to 1×10^5 colony forming units per mL. In 100 µL of medium, serial twofold dilutions of test compound were prepared. The test compound was tested in the range of 2000-8 µg mL^{-1}. Growth control with no test compound and sterility control were maintained during the experiment. Microtitre plate was incubated at 37°C for 24 hours.0.01% solution of resazurin dye ('Sigma Aldrich') was prepared and 30 µL was added in each well. Microtitre plate was again placed in incubator for 3-4 hours. A color change from blue to pink for resazurin dye was indicative of bacterial growth. Minimum concentration of test compound that inhibited the change in color was regarded as MIC value.

Effect of test compound on growth curve of *P. aeruginosa BDU49*

Turbidimetric method (Maia et al., 2016) was used to evaluate effect of various multiples of MIC of test compound (0.5×MIC, MIC and 2×MIC) on growth curve of *Pseudomonas aeruginosa BDU-49*.100 mL shaking flasks containing 50 mL Mueller-Hinton broth with appropriate concentration of test compound were inoculated with exponentially grown *Pseudomonas aeruginosa BDU-49* to yield final concentration of 1×10^6 colony forming units per mL. A growth control flask and sterility control flask were maintained during the experiment. All the flasks were incubated at 37°C in shaking incubator and aliquots were removed after every 60 min. until 8 hours. Optical density (OD) was measured by spectrophotometer at 600 nm. After 8 hours, 1 mL sample was taken from each flask and serially tenfold dilution were prepared and spread on Mueller-Hinton agar to demonstrate bacteriostatic or bactericidal effects of test compound. Growth curves were constructed by plotting OD value (Verticle axis) versus time (horizontal axis).

Time-kill curve determination against *P. aeruginosa BDU 49*

Broth macrodilution method was used to construct time kill curve according to the Clinical and Laboratory Standards Institute guidelines (Barry et al., 1999; Scoffone et al., 2015). Briefly, for time kill curve, various multiples of MIC of test compound (0.5×MIC, MIC and 2×MIC) were evaluated. 50 mL shaking flasks containing 30 mL Mueller-Hinton broth with appropriate concentration of test compound were inoculated with 24 hour freshly grown *Pseudomonas aeruginosa BDU-49* to yield final concentration of 1×10^6 colony forming units per mL. All the flasks were incubated at 37°C in shaking incubator and 1 mL sample from each flask was taken at 0, 2, 4, 6, 8 and 24 hours. Serial tenfold dilution were prepared in sterile normal saline and spread aseptically on Mueller-Hinton agar plates. All the agar plates were incubated at 37°C for 24 hours and colonies were counted to determine colony forming units per mL. Time kill curve was designed by plotting log 10 of colony forming units per mL (Verticle axis) versus time (horizontal axis).Bactericidal activity was demonstrated as decrease of 99.9% (⩾ 3 Log10) of total number of colony forming units per mL in the initial sample. Bacteriostatic activity was demonstrated as maintenance of original inoculum concentration or decrease of less than 99.9% (< 3 Log10) of total number of colony forming units per mL in the initial sample.

Results and discussion

Identification of a novel dialkyl carboxylate cyclohexane derivative

The targeted compound was synthesized by condensation of benzaldehyde, methyl-2-cyanoacetate and acetylacetone in the presence of sodium hydroxide. The product was obtained in quantitative yield. The structure of the obtained compound was investigated by ^{1}H and 13C NMR spectroscopy and elemental analysis. According to 1H NMR spectrum, the signals from methoxy groups are observed at 3.35 and 3.50 ppm, whereas aromatic hydrogens are in the range of 7.20-7.47 and 7.55-7.72 ppm. According to ^{13}C NMR spectrum, the signals from methoxygroups are observed at 66.75 and 72.81 ppm, whereas aromatic carbons are at 127.78-134.5 ppm. Ether group signals are observed at 160.5 and 162 ppm and carbonyl group signal is at 193.55 ppm.

In vitro antimicrobial activity of test compound

Table 1 shows the test compound exhibited variable antibacterial properties against different test pathogens. Generally, dialkyl carboxylate cyclohexane derivative was found to be more active against Gram-negative bacteria. *Pseudomonas aeruginosa BDU-49* was found to be the most susceptible bacteria (26.7, 19.3, and 16 mm zone of inhibition at 0.3, 0.1 and 0.05% concentration of test compound respectively) among all the tested cultures. Gram-positive bacteria were found to be resistant against test compound.

Results of agar well diffusion assay were validated by resazurin microplate assay. As shown in Table 1, *Pseudomonas aeruginosa BDU-49* was found to be most sensitive pathogen with MIC 62.5 μg mL^{-1}. For all Gram-positive bacteria, MIC was found to be > 2000.

Effect of test compound on growth curve of *P. aeruginosa BDU-49*

There are significant differences in growth curves of *Pseudomonas aeruginosa BDU-49* (Figure 4) when exposed to various concentrations of test compound. OD value represents total bacterial mass in the sample. At sub inhibitory concentration i.e. 0.5×MIC, there is minimal variation in the OD value when compared to control. At MIC concentration, difference in OD value was more evident after 4 hours. At 2×MIC concentration, inhibitory effects of test compound on growth of *Pseudomonas aeruginosa BDU-49* are evident even at start of growth curve. In the agar plates inoculated at end of experiment (8 hour), bacterial colonies were visible for 0.5×MIC and MIC concentration, while there were no colonies for 2×MIC concentration.

Time kill curve analysis

Bacterial colonies visible with naked eyes were counted to calculate colony forming units per mL. Log 10 of colony forming units per mL was plotted against time to construct time kill curves (Figure 5). Time kill assay exhibited that inhibitory effects and killing of *Pseudomonas aeruginosa BDU-49* by test compound were concentration dependent. There was little difference in time kill curves of bacteria at 0.5×MIC and control. At MIC concentration, number of bacteria was almost same after 24 hours as in original inoculum. There was significant decrease in the number of viable bacteria at 2×MIC concentration after 6 hours of the inoculation.

Table 1. *In vitro* antimicrobial activity of dimethyl-5-acetyl-1,3-dicyano-4-hydroxy-4-methyl-2,6-diphenylcyclohexane-1,3-dicarboxylate.

Test Cultures	Average diameter of inhibition zone(mm)			MIC (µg mL^{-1})
	Concentration of test compound			
	0.3%	0.1%	0.05%	
E.coli	18.3±0.1	14.7±0.2	-	250
K.pneumoniae	19.7±0.3	14±0	-	500
A.baumannii	17.7±0.2	13.7±0.4	-	125
P.aeruginosa	26.7±0.3	19.3±0.2	16±0	62.5
S.aureus	-	-	-	> 2000
B.subtilis	-	-	-	> 2000
B.megaterium	-	-	-	> 2000
B.mesentericus	-	-	-	> 2000

(-): Inactivity, *Data represents the mean values of three replicates.

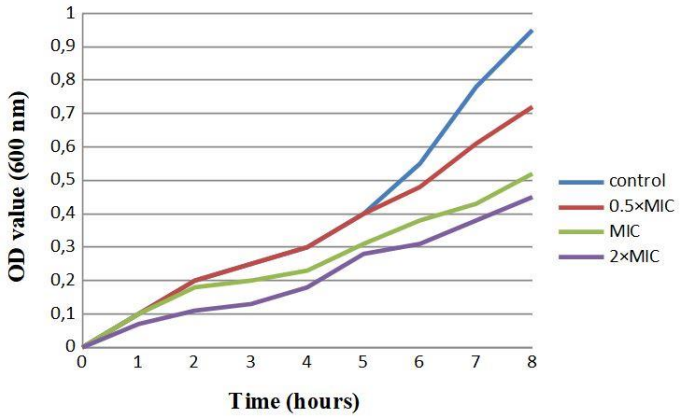

Figure 4. Effect of test compound on growth curve of *Pseudomonas aeruginosa* BDU-49.

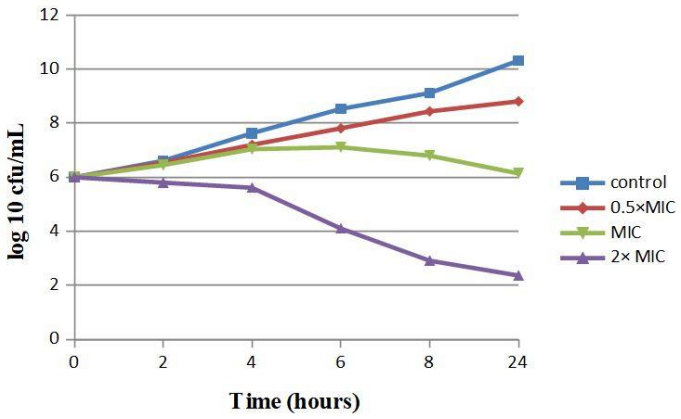

Figure 5. Time kill curve assay chart showing effect of test compound on *Pseudomonas aeruginosa BDU-49*.

It is worth mentioning that drug development = studies are not seriously considering drug discovery against respiratory = infections caused by *Pseudomonas aeruginosa* in patients suffering from cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), and ventilator-associated pneumonia (VAP – Folkesson et al., 2012; Peña et al., 2013). In the last decade, the emergence of multi drug resistant *Pseudomonas aeruginosa* strains is a major challenge in treatment and eradication of CF, COPD and VAP (Fricks-Lima et al., 2011). In this perspective, discovery of new substances effective against *Pseudomonas aeruginosa* is of paramount significance as it causes fatal infections.

In the current study, we reported that a new drug candidate, dialkyl carboxylate cyclohexane derivative exhibits promising antimicrobial activity against *Pseudomonas aeruginosa BDU-49* i.e. zone of inhibition and the MIC 26.7 mm and8 µg mL^{-1}, respectively. The compound has also exhibited antimicrobial properties against three other Gram-negative bacteria. Contrary to this, all the tested Gram-positive bacteria were found to be resistant against the test compound. Our results are supported by our previous studies (Shoaib et al., 2019c), which showed that ethyl, isopropyl and butyl carboxylate cyclohexane derivatives were more potent

against Gram-negative bacteria as compared to Gram-positive bacteria. Functionally substituted monocyclic and spirocyclic cyclohexane derivatives showed better antimicrobial activity against Gram-negative bacteria. These results are synchronized with our findings (Shoaib et al., 2020). = Toluenesulfonyl derivatives of pyrazoles annelated with polyfunctional cyclohexane ring also exhibited better antibacterial properties against Gram-negative bacteria as compared to Gram-positive bacteria (Ismiyev, Shoaib, Ganbarov, & Agayeva, 2019). These results also support and validate our findings.

Findings of (Shoaib, 2019d) are concurrent with our results who showed that, diethyl-4-hydroxy-4-methyl-2-(3nitrophenyl)-6-oxocyclohexane-1,3-dicarboxylate exhibited moderate activity against Gram-negative bacteria while Gram-positive bacteria were found to be resistant. = Benzofuran cyclohexane-5-carboxylate derivatives exhibited better antimicrobial activity against Gram-positive bacteria as compared to Gram-negative bacteria (Urzua et al., 2008). The contradiction with our findings is due to presence of dimethyl group in our compound. Inactivity against Gram-positive bacteria can be attributed to difference in the structure of cell wall. Antimicrobial profile of diethyl 6-oxo-3-(2-furyl)-2,4-dicyano-8-(di-ethylamino)bicyclo[3.2.1]octane-2,4-dicarboxylate and diethyl 6-oxo-8-(piperidine-1-yl)-3-(2-furyl)-2,4-dicyanobicyclo[3.2.1]octane-2,4-dicarboxylate contradicts our findings (Ismiyev et al., 2020). This is attributed to presence of bicyclo[3.2.1]octane fragment in the above mentioned compounds which led to their higher antimicrobial potential against Gram-positive bacteria.

The test compound has been shown to be most effective against *Pseudomonas aeruginosa BDU-49*, thus providing a good starting point for determining its effect on growth curve and time kill assay against *Pseudomonas aeruginosa BDU-49*. The growth curves of *Pseudomonas aeruginosa BDU-49* treated with different concentrations of test compound (0.5×MIC, MIC and 2×MIC) demonstrated that test compound could inhibit the growth and reproduction of bacteria. The growths of bacterial cells treated with MIC and 2×MIC were inhibited. The growth of the test pathogen treated with MIC was also a little less than that of bacteria in the control group. These results specify that the anti-*Pseudomonas* activity of MIC of test compound could hamper bacterial growth to some extent. MIC and 2×MICof test compound are required to reduce the reproduction and growth of the bacteria.

A standardized *in vitro* time kill curve assay was performed and the resulting information was used to study pharmacodynamics of test compound unfolding the association between the concentration of compound and the net growth rate of *Pseudomonas aeruginosa BDU-49*. Time kill assay provides more precise information concerning the effect of the test compound on bacteria since the measurements are taken over different times. Furthermore, this technique has ability to differentiate bactericidal activities and bacterial re-growth (Soudeiha, Dahdouh, Azar, Sarkis, & Daoud, 2017). At 0.5×MIC and MIC, test compound was found to be bacteriostatic. After 24 hour of treatment, number of viable bacteria was almost same as in original sample thus establishing bacteriostatic nature of test compound. At 2×MIC, test compound was found to be bactericidal, as there was reduction of 99.9% ($\geqslant$ 3 Log10) of total number of colony forming units per mL in original inoculum. The marked decrease in number of viable bacteria at 2×MICcould be explained by the fact that, test compound is supposed to be directed at DNA gyrase, thus inhibiting DNA synthesis (Silva, Lourenço, Queiroz, & Domingues, 2011). Thus, test compound is bactericidal at 2×MIC and bacteriostatic at MIC and <MIC concentrations.

Conclusion

The present study demonstrated the antimicrobial properties of novel dialkyl carboxylate cyclohexane derivative. Test compound showed better antimicrobial activity against Gram-negative bacteria and *Pseudomonas aeruginosa BDU-49* was found to be the most sensitive test culture. The growth curves of *Pseudomonas aeruginosa BDU-49* exhibited that test compound has positive effect on inhibition of the growth and reproduction of bacteria. Test compound was found to be bactericidal at higher concentration, while effect was bacteriostatic at MIC. = Thus, dialkyl carboxylate cyclohexane derivative could at as potential anti-*Pseudomonas aeruginosa* compound in the future.

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