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ABSTRACT

of the dissertation for the degree of Doctor of Philosophy

ANTIMICROBIAL ACTIVITY OF FUNCTIONALLY SUBSTITUTED CYCLOHEXANE COMPOUNDS

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INTRODUCTION

Actuality of research. The spread of antimicrobial resistance is the most grave and perpetual threat to human life and *"requires constant unearthing of new antimicrobial drugs for successful management of infectious ailments"*[1]. Multidrug resistant bacteria are increasing at an unprecedented rate. These microorganisms are major obstacle in treatment and eradiation of infectious diseases. A limited number of antibiotics are effective against Gram-positive bacteria and Gram-negative bacteria have acquired resistance to almost all available antimicrobial agent. "*Due to increasing antimicrobial resistance, antibiotics are becoming less valuable and effective*"[2]. It is direly required to formulate novel antimicrobial compounds, especially against drug resistant bacteria, multidrug resistant bacteria and pan-drug resistant bacteria.

World Health Organization (WHO) has accentuated the "*need of taking wide spread action against multidrug resistant pathogens by developing new antimicrobial drugs*"[3] with unique mode of action. Dearth of newly developed antimicrobial drugs and rapid development of antimicrobial resistance pose serious challenge in health care systems. Due to this situation, scientists are exploring novel antimicrobial compounds from a variety of origins as new antimicrobial substances.

Currently, there is an increasing interest in the "*synthesis and evaluation of organic compounds as economical, feasible and probable antimicrobial agents of future"*[4]. Different strategies have

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[1]Saleh, S.S. Biological activity Study for some heterocyclic compounds and their impact on the gram positive and negative bacteria / S.S. Saleh, S.A. Siham, A.M. Israa [et al.] // Energy Procedia, – 2019, 157, – p. 296–306.

[2]Theuretzbacher, U. Global antibacterial resistance; The never-ending story // Journal of Global Antimicrobial Resistance, – 2013, 1(2), – p.63–69.

[3]Suraj, N. Antimicrobial and Antibiotic Resistance / N. Suraj, P. Mali, M. Sapkal // International Journal of Pharmacy and Pharmaceutical Research, – 2019, 4(1), – p. 184–189.

[4]Rani V.L. Synthesis and Antimicrobial Activity of Novel Pyrazole-5-one Containing 1, 3, 4-oxadiazole Sulfonyl Phosphonates / V.L. Rani, K. Ravindranath // American Journal of Organic Chemistry, – 2016, 6(1), – p. 1–7.

been designed to deal with the dilemma of antimicrobial resistance. Organic compounds containing heterocyclic ring systems are center of attention for new antimicrobial drugs because of their large variety of biological actions. Heterocyclic compounds have great significance in organic synthetic routes because subtle modifications in these compounds alter their reactivity. These compounds have "*strong physicochemical properties due to diversity in type and size of ring structures and substituent groups of the core scaffold*"[5]. Hybrid organic compounds which coalesce two or more biologically active substances and functional substitution of organic compounds lead to exponential increase in antimicrobial activity of these compounds. Functionally substituted derivatives of different organic compounds like quaternary ammonium compounds, pyrazolone, thiones, isatin, indole, piperidine, benzoxazolinone etc. are superlative existing compounds with prospective antimicrobial profile. These derivatives have distinctive mechanism and are not affected by bacterial resistance methods. This, in turn accounts for their better antimicrobial profile. These compounds have demonstrated various "*pharmacological properties like antibacterial, antifungal, antidepressant and antihistamines*"[6]. Organic compounds having quinone moiety continue to serve as base for synthesis of wide range of novel compounds. These derivatives are applied as drugs, herbicides, fungicidal agents and bactericidal compounds. "*Due to multiple bonding possibilities and donor atoms such as oxygen, nitrogen and sulfur, synthetic organic derivatives have diverse biological properties and are used as antibacterial, antifungal, anti-tubercular, antithyroid, anthelmintic agents*"[7].

[5]Gomtsyan, A. Heterocycles in drugs and drug discovery // Chemistry of Heterocyclic Compounds, – 2012, 48, – p. 7–10.

[6]Al-Refai, M. One-pot synthesis and antimicrobial activity of new 4,6-disubstituted-3,4-dihydropyrimidine-2(1H)thiones / M. Al-Refai, M. Ibrahim, A. Al-Fawwaz [et al.] // Chemistry–A European Journal, – 2017, 8(1), – p. 96-100.

[7]Arslan, H. Antimicrobial Activity of Some Thiourea Derivatives and Their Nickel and Copper Complexes / H. Arslan, N. Duran, G. Borekci [et al.] // Molecules, – 2009, 14, – p. 519 –527.

Functionally substituted derivatives of cyclohexane are unique substances with undetermined and hidden potential antibacterial and antifungal properties. Due to their cyclic ring structures, functionally substituted cyclohexane derivatives have diverse biological properties including "*anticancer activity*"[8], "*antioxidant activity"*[9], "*cytotoxic activity"*[10], "*analgesic activity"*[11] and "*anti-inflammatory activity"*[12]. Therefore, it is necessary to unearth novel and superior antimicrobial compounds to combat hitch of antimicrobial resistance. Although the different biological properties of functionally substituted cyclohexane derivatives have been deliberated, yet there is no inclusive study present to elucidate the antimicrobial potential of novel functionally substituted cyclohexane derivatives. Physical and chemical properties of functionally substituted cyclohexane compounds synthesized by chemist at Baku State University have been studied, but their biological properties are not explored. Therefore, it is imperative to explore the antimicrobial profile of functionally substituted cyclohexane compounds.

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[8]Song, L. Dimedone derivative {2-[(4-hydroxy-phenylamino)-methylene]-5, 5-dimethyl-cyclohexane-1, 3-dione} plays an important role in breast cancer treatment / L. Song, H. Kang, D. Liu [et al.] // Tropical Journal of Pharmaceutical Research, – 2015, 14, – p.1719–1722.

[9]Flefel, E.M. Pharmacological evaluation of some novel synthesized compounds derived from spiro (cyclohexane-1, 2′-thiazolidines / E.M. Flefel, H.H. Sayed, A.I. Hashem [et al.] // Medicinal Chemistry Research, – 2014, 23, – p. 2515–2527.

[10]Shoaib, M. Cyclohexane and its functionally substituted derivatives: important class of organic compounds with potential antimicrobial activities / M. Shoaib, A.A. Israyilova, G.K. Ganbarov // Journal of Microbiology, Biotechnology and Food Sciences, – 2019, 9(1), – 84–87.

[11]Amin, K.M. Synthesis, biological evaluation and molecular docking of novel series of spiro[(2H,3H) quinazoline-2,1-cyclohexan]-4 (1H0-one derivatives as anti-inflammatory and analgesic agents / K.M. Amin, M.M. Kamel, M.M. Anwar [et al.] // European Journal of Medicinal Chemistry, – 2010, 45, – p. 2117 –2131.

[12]Usegilo, M. Synthesis of 3H-spiro [benzofuran-2, 1′-cyclohexane] derivatives from naturally occurring filifolinol and their classical complement pathway inhibitory activity / M. Usegilo, P.M. Castellano, M.A. Operto [et al.] // Bioorganic medicinal Chemistry Letters, – 2006, 16, – p. 5097–5101.

Purpose of research. The major purpose of the study undertaken was to examine the potency of functionally substituted cyclohexane derivatives against conditionally pathogenic Gram-positive bacteria, Gram-negative bacteria and yeast. This purpose was achieved through following steps;

- Screening of fifty functionally substituted cyclohexane derivatives against conditionally pathogenic Gram-positive bacteria, Gram-negative bacteria and yeast by agar well diffusion assay.
- Evaluation of minimum inhibitory concentration (MIC) of test compounds against conditionally pathogenic Gram-positive bacteria and Gram-negative bacteria by resazurin microplate assay.
- Determination of effect of highly active test compounds on growth curves of susceptible test cultures.
- Evaluation of highly active test compounds for time-kill curve determination of bacteriostatic or bactericidal nature of test compounds.

Research methods. For research, initially agar well diffusion assay was used to screen antimicrobial properties of functionally substituted cyclohexane derivatives against conditionally pathogenic Gram-positive bacteria, Gram-negative bacteria and yeast. Minimum inhibitory concentration was determined by resazurin dye method against those compounds which showed activity by agar well diffusion assay against conditionally pathogenic Gram-positive bacteria and Gram-negative bacteria. For compounds which were highly active, growth curves were constructed to see the effects of functionally substituted cyclohexane compounds on growth and reproduction of susceptible conditionally pathogenic Gram-positive bacteria and Gram-negative bacteria. Finally, time kill assay was used to unearth bacteriostatic and bactericidal nature of functionally substituted cyclohexane compounds.

Main provisions submitted for defense.

- Functionally substituted cyclohexane compounds showed variable antimicrobial properties against different

pathogens. Gram-negative bacteria were found to be the most susceptible, while yeasts were found to be most resistant test cultures.

- Ethyl 2-(4-(2-ethoxy-2-oxoethyl)thiazol-2-yl)-6-hydroxy-6-methyl-3- oxo-4- (p-tolyl)-2,3,4,5,6,7- hexahydro -1H-indazole -5-carboxylate and ethyl-4-methyl-6-phenyl-2-oxocyclohex-3-en-1-carboxylate exhibited least minimum inhibitory concentration value of 31.25 µg/ml against *Acinetobacter baumannii* BDU-32 and *Klebsiella pneumoniae* BDU-44 respectively.
- Effect of functionally substituted cyclohexane compounds on growth curves of test cultures showed that these compounds have significant potential to inhibit growth and reproduction of test cultures.
- Antimicrobial activity of test compounds against different pathogens was found to be concentration dependent. Functionally substituted cyclohexane compounds were found to be bacteriostatic at concentration $\leq$ minimum inhibitory concentration and bactericidal at concentration greater than minimum inhibitory concentration.

Scientific novelty of research. Antimicrobial activity of fifty functionally substituted cyclohexane derivatives against conditionally pathogenic Gram-positive bacteria, Gram-negative bacteria and yeast (genus *Candida*) was studied for the first time. These derivatives showed variable antimicrobial activities. Some of these compounds showed considerable antimicrobial activity against bacteria, while other compounds demonstrated notable antimicrobial properties against fungal species. It is noteworthy that most of the tested substances showed better antimicrobial activity against Gram-negative bacteria as compared to Gram-positive bacteria. *Acinetobacter baumannii* BDU-32 was found to be the most sensitive test culture among all the tested Gram-negative bacteria, while *Staphylococcus aureus* BDU-23 was found to be the most sensitive test culture among all the tested Gram-positive bacteria. Six compounds were found to be highly effective against different test cultures. Maximum diameter of zone of inhibition was found up to

30 mm. Least minimum inhibitory concentration value was found to be 31.25µg/ml. It was shown that the above mentioned test compounds significantly inhibited growth and reproduction of test cultures. Functionally substituted cyclohexane compounds had bacteriostatic effects at minimum inhibitory concentration values against test cultures and bactericidal effects at concentrations greater than minimum inhibitory concentration values.

Theoretical and practical significance of research. Our results can enrich our knowledge about antimicrobial activity of synthetic functionally substituted cyclohexane compounds. Some of functionally substituted cyclohexane compounds exhibited antimicrobial activity against only Gram-negative bacteria, thus these substances can act as selective antimicrobial agents. Ethyl-6-hydroxy-6-methyl-3-oxo-4- phenyl- 1,3,4,5,6,7- hexa hydrobenzo [c] isoxazole- carboxylate is a supreme example of such compound with antimicrobial properties against selective bacteria (Patent No. İ 2020 0097). Some of functionally substituted cyclohexane compounds exhibited antimicrobial activity against both Gram-negative bacteria and Gram-positive bacteria. Hence, these compounds could act as potential disinfectants. Ethyl -4- phenyl-6- (p-tolyl)-2- dicyano methylene cyclohex -3-en-1-carboxylateis one of such compounds with above mentioned potential. It was found that ethyl 2-(4-(2-ethoxy-2-oxoethyl) thiazol -2- yl) -6- hydroxy -6- methyl-3-oxo-4-(p-tolyl)-2,3,4,5,6,7-hexahydro-1H-indazole-5-carboxylate was the most active agent against *Acinetobacter baumannii BDU-32* (having average zone of inhibition and minimum inhibitory concentration 24.7±0.2 mm at 0.3% concentration and 31.25 µg/ml, respectively). *Escherichia coli BDU-12*was found to be the most sensitive against diethyl 2,4-dicyano-3-(4-chlorophenyl)-8-morpholino-6-oxobicyclo [3.2.1]octane-2,4-dicarboxylate (having average zone of inhibition and minimum inhibitory concentration 28.3±0.2 mm at 0.3% concentration and 62.5 µg/ml, respectively). Among all test compounds, ethyl -4- methyl -6- phenyl -2- oxo cyclohex -3- en-1-carboxylate was the strongest antibacterial agent against *Klebsiella pneumoniae BDU-44* (having average zone of inhibition and minimum inhibitory concentration 25.7±0.6 mm at 0.3%

concentration and 31.25 µg/ml, respectively). *Pseudomonas aeruginosa BDU-49* was the most susceptible among all the test cultures against dimethyl 5-acetyl-1,3-dicyano-4-hydroxy-4-methyl-2,6-diphenylcyclohexane-1,3-dicarboxylate (having average zone of inhibition and minimum inhibitory concentration 26.7±0.3 mm at 0.3% concentration and 62.5 µg/ml, respectively). 2-(1-acetyl-5-cyano-6-(2,4-dichlorophenyl)-2-methyl-4-oxo-3-azabicyclo [3.1.0] hexan -2-yl) malononitrile was found to be most active antibacterial agent against *Staphylococcus aureus BDU-23* (having average zone of inhibition and minimum inhibitory concentration 20.3±0.3 mm at 0.3% concentration and 62.5 µg/ml, respectively). Thus, these functionally substituted cyclohexane derivatives could act as potential antimicrobial drugs against different pathogens in future.

Personal contribution of applicant. In carrying out all the experiments, PhD student played the main role.

Publication, aprobation and application of the dissertation. Total 15 articles (10 research papers, 4 conference materials and 1 patent) related to the dissertation have been published. 3 research articles have been published in impact factor journals included in web of science. Dissertation materials are presented at the scientific conferences i.e. "9thInternational conference on innovative approaches in modern biology" (Baku, 2019), “International scientific and practical conference; protection and rational use of natural resources of the South Aral Sea Region” (Nukus, 2020), “XXV International Scientific and Practical Conference; Scientific Dialogue: Issues of Medicine" (St. Petersburg, 2020) and “International Scientific and Practical Conference; Science of Russia: Goals and Tasks” (Yekaterinburg, 2020).

Name of organization where dissertation work was carried out. Baku State University, Azerbaijan.

Structure of dissertation. Dissertation contains introduction, 6 chapters, discussions, conclusion and references. There are 15 tables and 24 figures included in dissertation. First chapter contains 19 pages; second chapter contains 21 pages, 3 tables and 1 figure; third chapter contains 32 pages, 3 tables and 9 figures; fourth chapter contains 9 pages, 2 tables and 2 figures; fifth chapter contains 10

pages and 6 figures; sixth chapter contains 13 pages, 6 tables and 6 figures and discussions consist of 19 pages. Dissertation consists of 173 pages, 218 references and 233882 characters.

CHAPTER I
FUNTIONALL SUBSTITUED ORGANIC COMPOUNDS AS ANIMICROBIAL SUBSTANCES

Section 1.1 and 1.2 of the dissertation consider data about development of antimicrobial resistance, mechanisms of antimicrobial resistance and drug resistant pathogens which accentuate the need of formulation of new antimicrobial drugs.

Section 1.3 of the dissertation highlights the importance of organic compounds as probable biological agents. Section 1.4 of the dissertation describes mechanism of action of synthetic organic substances.

Section 1.5 and 1.6 of the dissertation show antimicrobial profile of different synthetic organic compounds. Section 1.7 of the dissertation explains different properties of cyclohexane.

Last four sections of the dissertation describe biological profile of different types of cyclohexane derivatives. These include diamine derivatives, cyclohexyl methyl derivatives and miscellaneous derivatives of cyclohexane.

CHAPTER II
MATERIALS AND METHODS

Nutrient agar and Mueller-Hinton agar were used to cultivate bacteria, while sabouraud dextrose agar was used to cultivate yeast. Antimicrobial activity of fifty functionally substituted cyclohexane compounds was determined against four Gram-negative bacteria (*Escherichia coli* BDU-12, *Klebsiella pneumoniae* BDU-44, *Acinetobacter baumannii* BDU-32 and *Pseudomonas aeruginosa* BDU-49), four Gram-positive bacteria (*Staphylococcus aureus* BDU-23, *Bacillus Subtilis* BDU-50, *Bacillus mesentericus* BDU-51 and *Bacillus megaterium* BDU-N20) and three fungi (*Candida tropicalis* BDU LK30, *Candida pelliculosa* BDU KT55 and *Candida pseudotropicalis* BDU MA88). Fifty functionally substituted

cyclohexane compounds were obtained from Department of Organic chemistry, Baku State University Azerbaijan. Initially, all the test cultures were screened against test compounds by "*agar well diffusion technique*"[13]. Those compounds which showed activity by agar well diffusion technique were evaluated to determine minimum inhibitory concentration by "*resazurin dye method*"[14]. Six highly active compounds were selected and effect of these compounds on the "*growth curves of susceptible test cultures was determined by turbidimetric method*"[15]. "*Broth macrodilution method*"[16] was used to establish bacteriostatic or bactericidal nature of highly active test compounds by constructing time kill curves.

CHAPTER III
ANTIMIROBIAL SCREENING OF FUNTIONALLY SUBSTITUED CYCLOHEXANE COMPOUNDS

From the obtained results of agar well diffusion technique, it is evident that all functionally substituted cyclohexane derivatives showed variable antimicrobial activities (table 3.1). It is noteworthy that the most of the tested derivatives manifested stronger antimicrobial profile against Gram-negative bacteria than Gram-positive bacteria. Functionally substituted cyclohexane compounds exhibited better antibacterial properties than antifungal properties. Some of the functionally substituted cyclohexane substances were

[13]Balouiri, M. Methods for *in vitro* evaluating antimicrobial activity; A review / M. Balouiri, M. Sadiki, S.K. Ibnsouda // Journal of Pharmaceutical Analysis, – 2016, 6, – p. 71–79.

[14]Israyilova, A. Biochemical characterization of glutamate racemase—a new candidate drug target against *Burkholderia cenocepacia* infection / A. Israyilova, S. Buroni, F. Forneris [et al.] // PloS one, – 2016. 11(11), – p. 1–17.

[15]Maia, M.R. Simple and versatile turbidimetric monitoring of bacterial growth in liquid cultures using a customized 3D printed culture tube holder and a miniaturized spectrophotometer: application to facultative and strictly anaerobic bacteria / M.R. Maia, M. Sara, R.J.C. Ana // Frontiers in Microbiology, – 2016, 7, – p. 1381–1386.

[16]Scoffone, V.C. Efflux-mediated resistance to a benzothiadiazol derivative effective against Burkholderia cenocepacia / V.C. Scoffone, R. Olga, M. Vadim [et al.] // Frontiers in Microbiology, – 2015, 6, – p. 1–8.

noted to be highly effective against only Gram-negative bacteria, while Gram-positive bacteria were found to be resistant. This showed their selective potential against Gram-negative bacteria.

Escherichia coli BDU-12 was found to be moderately sensitive against functionally substituted cyclohexane derivatives. Diethyl 2,4-dicyano-3-(4-chlorophenyl)-8-morpho lino-6- oxo bicyclo [3.2.1] octane -2,4- dicarboxylate showed the strongest activity (average zone of inhibition 28.3 mm at 0.3% concentration, 17.3 mm at 0.1% concentration, and 13.7 mm at 0.05% concentration) against *Escherichia coli* BDU-12. Ten compounds were found to be completely inactive against *Escherichia coli* BDU-12.

Klebsiella pneumoniae BDU-44 was found to be more resistant to test compounds as compared to *Escherichia coli* BDU-12. Among all the tested compounds, 15 compounds were completely inactive against *Klebsiella pneumoniae BDU-44*. Diiso propyl 2,4- di cyano -8- (diethyl amino) -3- (2- furyl) -6-oxo bicyclo [3.2.1] octane -2,4 -dicarboxylate was potent antibacterial agent (average diameter of zone of inhibition 28.7 mm at 0.3% concentration, 19.7 mm at 0.1% concentration, and 14.3 mm at 0.05% concentration) against *Klebsiella pneumoniae BDU-44*.

Acinetobacter baumannii BDU-32 was recorded to be the most vulnerable test culture among all the Gram-negative bacteria.
Only 4 functionally substituted cyclohexane derivatives were completely inactive against *Acinetobacter baumannii BDU-32*. Maximum anti *Acinetobacter baumannii BDU-32* activity (average diameter of zone of inhibition 27.3 mm at 0.3% concentration and 14.7 mm at 0.1% concentration) was observed for ethyl -3-(allyl amino) -9,9- di methyl -7,11- di oxo- 1,5-di phenyl spiro [5.5] undec -2-ene-2-carboxylate. Diiso propyl 2,4- dicyano -8- (diethyl amino) -3-(2-furyl) -6-oxo bicyclo [3.2.1] octane-2,4 - dicarboxylate also exhibited considerable antibacterial activity against *Acinetobacter baumannii BDU-32*.

Pseudomonas aeruginosa BDU-49 was the most resistant test culture among all the Gram-negative bacteria. Majority of the tested functionally substituted cyclohexane derivatives were active against *Pseudomonas aeruginosa BDU-49* at only 0.3% concentration.

Table 3.1

Inhibition zone (mm) at 0.3% concentration of test compounds

No	*E. coli*	*K.pneumoniae*	*A. baumannii*	*P. aeruginosa*
1	17.3±0.4	18±0.4	27.3±0.2	17.7±0.3
2	17.7±0.4	20±0.8	24±0.4	15.3±0.3
3	18±0.2	16±0.2	25.3±0.5	14.3±0.2
5	18±0.3	21.3±0.1	24.7±0.2	14.7±0.1
6	24.3±0.3	24±0	19.7±0.3	15.3±0.3
8	28.3±0.2	20±0	18±0.2	15.7±0.4
19	24.7±0.1	21±0.3	19.3±0.2	14.5±0.4
24	18.3±0.1	19.7±0.3	17.7±0.2	26.7±0.3
32	16±0	25.7±0.6	16.7±0.2	15±0
34	16.3±0.2	28.7±0.3	20.7±0.3	17±0.3
46	16.3±0.4	17.3±0.6	22.3±0.3	19.7±0.2
50	25.3±0.3	17±0	19.7±0.1	12.3±0.4
No	***S. aureus***	***B. subtilis***	***B.megaterum***	***B.mesentricus***
6	26.7±0.7	15.3±0.3	14.3±0.3	16.7±0.7
9	20.3±0.3	19.3±0.2	18.7±0.3	16±0.3
16	15±0	18.7±0.5	17.3±0.3	18±0
19	16±0.3	19.7±0.2	18.3±0.1	15.7±0.6
41	17±0	17.7±0.3	18±0.3	18±0
42	12.3±0.6	18±0	19.7±0.1	19±0.3

No	*C. tropicalis*	*C. pelliculosa*	*C. pseudotropicalis*
8	21.7±0.2	21.3±0.4	22.7±0.6
9	16±0	15.7±0.8	21.7±0.1
24	17.7±0.3	15±0	22.3±0.8
31	15±0	17.3±0.2	13.3±0.6
32	18±0.3	15±0.1	17.3±0.2
34	17.7±0.2	15.3±0.3	18.3±0.2
44	23.7±0.8	17±0	24±0
45	23.3±0.3	18±0	21.7±0.4
46	20.3±0.2	23.7±0.3	30±0.6
48	15±0.3	19.7±0.4	20.3±0.2
49	20±0	22±0	20.7±0.6

50	22.3±0.3	17±0	20±0.3

Pseudomonas aeruginosa was found to be resistant for 13 test derivatives, while most of other test compounds exhibited weak to moderate activities. Dimethyl -5-actyl-1,3- dicyano -4- hydroxy -4-methyl -2,6 - diphenyl cyclohexane-1,3-dicarboxylate was observed to have most potent (average diameter of zone of inhibition 26.7 mm at 0.3% concentration, 19.3 mm at 0.1% concentration and 16 mm at 0.05% concentration) activity against *Pseudomonas aeruginosa BDU-49*.

Staphylococcus aureus BDU-23 was the most resistant test culture among all the Gram-positive bacteria. Half of the tested functionally substituted cyclohexane derivatives were found to be inactive against *Staphylococcus aureus BDU-23*. Majority of compounds exhibited weak to modest antibacterial properties only at 0.3% concentration. Ethyl -4- phenyl -6- (p-tolyl)-2-dicyano methylene cyclohex -3-en-1-carboxylate showed maximum antibacterial activity (average inhibition zone 26.7 mm at 0.3% concentration and 15.5 mm at 0.1% concentration) against *Staphylococcus aureus BDU-23*.There was only one compound i.e. 2-(1-acetyl-5-cyano-6-(2,4-dichlorophenyl)-2-methyl-4-oxo-3-aza bicyclo [3.1.0] hexan-2- yl)malononitrile which inhibited the growth of *Staphylococcus aureus BDU-23* at 0.05% concentration having average diameter of zone of inhibition 13.7 mm.

Functionally substituted cyclohexane derivatives were moderately active against *Bacillus species*. Among the test compounds, 17 compounds showed activity against none of the *Bacillus species*. *Bacillus mesentericus BDU-51* was observed to be the most vulnerable among the tested *Bacillus species*, while *Bacillus Subtilis BDU-50* was the most resistant against the tested derivatives. 5-Actyl -6-hydroxy -3,6- di methyl-4- phenyl-1-tosyl-4,5,6,7-tetrahydro-1Hindazol exhibited maximum activity (inhibition zone 27 mm at 0.3% concentration and 18 mm at 0.1% concentration) against *Bacillus mesentericus BDU-51*.Ethyl-4-methyl-6-phehyl-2-dicyano methylene cyclohex-3-en-1-carboxylate was noted to be the strongest compound against *Bacillus megaterium BDU-N20* having average diameter of zone of inhibition 20.3 mm at 0.3%

concentration and 15.3 mm at 0.1% concentration. Against *Bacillus Subtilis BDU-50*, ethyl 3- (propylamino)-9,9-dimethyl -7,11-di oxo-1,5-di (p-tolyl) [5.5] undec-2-ene-2-carboxylate was the most strongest antibacterial agent with average inhibition zone 19.7 mm at 0.3% concentration and 15.3 mm at 0.1% concentration.

Functionally substituted cyclohexane compounds showed variable activity against different *Candida* species. *Candida pseudotropicalis* BDU MA88 was found to be the most sensitive fungi, while *Candida pelliculosa* BDU KT55 was observed as the most resistant fungi.

CHAPTER IV
ASSESSMENT OF MINIMUM INHIBITORY CONCENTRATION (MIC) OF TEST COMPOUNDS

Overall, minimum inhibitory concentrations (MIC) of functionally substituted cyclohexane compounds (which showed better activity by agar well diffusion method) ranged from 2000µg/ml to 62.5µg/ml (table 4.1). MIC values for Gram-positive bacteria were found to be higher as compared to Gram-negative bacteria, which validates our findings of agar well diffusion technique. Broth microdilution method using resazurin dye showed that diethyl- 2,4- di cyano-3-(4-chlorophenyl)-8-morpho lino-6-oxo bicyclo [3.2.1]octane-2,4-dicarboxylate and diethyl 4-hydroxy-4-methyl-6-(2-(4-methylphenylsulfonamido) ethyl amino) cyclohex-1-en-1,3-dicarboxylate were the most strong agents with lowest MIC (62.5µg/ml for both compounds) against *Escherichia coliBDU-12*. For majority of functionally substituted cyclohexane derivatives, MIC values were observed between 500 and 1000µg/ml against *Escherichia coli*. Ethyl -4-methyl -6-phenyl-2-oxocyclohex-3-en-1-carboxylate demonstrated lowest MIC value of 31.25µg/ml against *Klebsiella pneumoniae BDU-44*.

For majority of test compounds, MIC values were noted between 250 and 1000µg/m against *Klebsiella pneumoniae BDU-44.Acinetobacter baumannii BDU-32* was the most sensitive test culture. For ethyl 2-(4-(2- ethoxy-2 -oxo ethyl) thiazole-2-yl)-6-hydroxy-6-methyl-3-oxo-4- (p-tolyl)-2,3,4,5,6, 7-hexa hydro-1H indazol-5-carboxylate, lowest MIC value of 31.25µg/ml was noted

against *Acinetobacter baumannii BDU-32*. For majority of test compounds, MIC values were found to be between 250 and 500µg/ml against *Acinetobacter baumannii BDU-32*.*Pseudomonas aeruginosa BDU-49* was the most resistant test culture. Dimethyl 5-acetyl-1,3-dicyano-4-hydroxy-4-methyl-2,6-diphenyl cyclohex ane-1,3-dicarboxylate was the most active agent against *Pseudomonas aeruginosa BDU-49* with MIC value of 62.5µg/ml. For majority of test compounds, MIC against *Pseudomonas aeruginosa BDU-49* was found to be greater than 1000 µg/ml.

Table 4.1

Minimum inhibitory concentrations (µg/ml) of test compounds

No	***E. coli***	***K.pneumoniae***	***A. baumannii***	***P. aeruginosa***
2	125	125	250	1000
5	250	125	31.25	500
6	250	250	125	500
8	62.5	250	250	500
19	62.5	125	500	1000
24	1000	1000	1000	62.5
27	125	125	125	1000
32	500	31.25	500	1000
34	1000	62.5	500	1000
50	125	1000	500	2000
No	***S. aureus***	**B. subtilis**	***B.megaterum***	***B.mesentricus***
5	500	250	250	250
7	250	125	125	125
9	62.5	125	250	250
16	500	250	250	250
18	250	1000	500	500

It was noted that 2-(1-acetyl-5-cyano-6-(2,4-dichlorophenyl)-2-methyl-4-oxo-3-aza bicyclo [3.1.0]hexan -2-yl)malononitrile was the strongest antibacterial agent against *Staphylococcus aureus BDU-23* with MIC of 62.5µg/ml. For majority of test compounds, MIC against *Staphylococcus aureus BDU-23* was found to be greater than 2000µg/ml. All the three tested *Bacillus species* were noted to be

resistant against test compounds. *Bacillus megaterium BDU-N20* was found to be the most resistant, while weak to modest antibacterial profile was observed against *Bacillus mesentericus BDU-51* and *Bacillus Subtilis BDU-50*. Diethyl -9-hydroxy-9-methyl- 7-phenyl-1,4-diaza spiro[4.5]decane-6,8- dicarboxylate and 2-(1-actyl-5-cyano-6-(2,4-dichloro phenyl)-2- methyl- 4- oxo -3-aza bi cyclo [3.1.0]hexan - 2 - yl)malononitrile were the most strong antibacterial compounds against *Bacillus Subtilis BDU-50* having MIC value of 125µg/ml. Diethyl -9 -hydroxy-9- methyl -7-phenyl -1,4-diaza spiro[4.5]decane-6,8-dicarboxylate was the most active test compound against *Bacillus megaterium BDU-N20* and *Bacillus mesentericus BDU-51* having MIC value of 125µg/ml. For majority of test compounds, MIC against *Bacillus Subtilis BDU-50*, *Bacillus megaterium BDU-N20* and *Bacillus mesentericus BDU-51*was found to be greater than 2000µg/ml.

CHAPTER V
EFFECT OF TEST COMPOUNDS ON GROWTH CURVES OF BACTERIA

Generally, growth inhibiting effects were noted to be better for Gram-negative bacteria than Gram-positive bacteria. The growth curves of *Acinetobacter baumannii BDU-32* treated with 15.63, 31.25 and 62.5 µg/ml ethyl 2-(4-(2- ethoxy-2-oxo ethyl) thiazole-2-yl)-6-hydroxy-6-methyl -3-oxo-4- (p-tolyl) -2,3,4,5,6,7-hexa hydro-1H indazol-5-carboxylate pointed out that test compound could hinder the growth and reproduction of test culture (figure 5.1). After 4 hours, almost all bacterial cells treated with 2×MIC (62.5 µg/ml) were dead. The growth of *Acinetobacter baumannii BDU-32* treated with 0.5×MIC (15.63 µg/ml) of test compound was also slightly lesser than that of cells in the control group. Significant inhibition of reproduction of *Acinetobacter baumannii BDU-32* was observed in the group treated with MIC (31.25 µg/ml) concentration of substance.

The difference in the optical density values of the control group and treated group increases by increasing the concentration of the test substance. The growth curves of *Escherichia coli BDU-12*

exposed with 31.25, 62.5 and 125 µg/ml of diethyl-2,4-di cyano -3-(4-chlorophenyl)-8-morpho lino-6-oxo bicyclo [3.2.1]octane -2,4-dicarboxylate that test compound caused an obvious delay in growth of *Escherichia coli BDU-12*. Considerable inhibition of proliferation of *Escherichia coli BDU-12* was noted in the group treated with MIC (62.5 µg/ml) concentration of test derivative. With increase in the concentration of the substance, inhibitory effect on bacterial growth was accentuated. The growth curves of *Klebsiella pneumoniae BDU-44* treated with 15.63, 31.25 and 62.5 µg/ml of ethyl -4-methyl -6-phenyl-2- oxo cyclohex-3-en-1-carboxylate exhibited that different concentrations of test compound had huge potential to restrain the growth and reproduction of test bacteria.

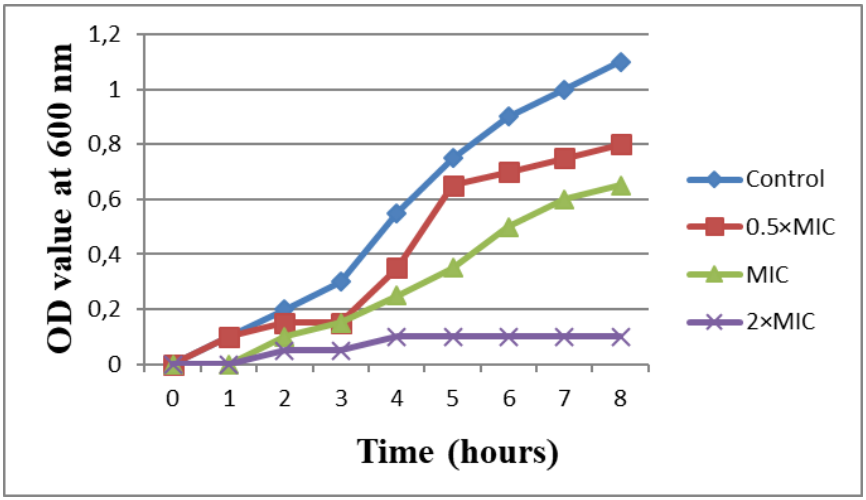

Figure 5.1. Growth curves of *Acinetobacter baumannii BDU-32* treated with different concentrations of test compound at standard conditions.

Periodic measurements of optical density value showed that after 7 hours, bacterial cells exposed with 2×MIC (62.5 µg/ml) manifested arrest of growth and reproduction. After 8 hours, difference in the optical density values of bacterial cells treated with 2×MIC (62.5 µg/ml) and bacterial cells in control group was greater than 1. The growth of bacterial cells treated with MIC (31.25 µg/ml)

concentration of test derivative was significantly delayed. Growth curves of *Pseudomonas aeruginosa BDU-49* exposed with 31.25 and 62.5 and 125 µg/ml of dimethyl 5- acetyl -1,3- dicyano -4- hydroxy -4-methyl-2,6-diphenyl cyclohexane-1,3-dicarboxylate showed the varying degree of delay in growth of test culture.

At 2×MIC concentration, inhibitory effects of test compound on growth of *Pseudomonas aeruginosa BDU-49* are evident even at start of growth curve. The growth curves of *Staphylococcus aureus BDU-23* treated with 2-(1-acetyl-5-cyano-6-(2,4-dichlorophenyl)-2- methyl -4-oxo-3- aza bicyclo [3.1.0] hexan-2-yl)malononitrile demonstrated that test compound could hamper the growth and reproduction of test culture. The optical density value of *Staphylococcus aureus BDU-23*treated with 2×MIC was almost half as compared to untreated group.

CHAPTER VI
TIME KILL CURVE ASSAY FOR *IN VITRO* EVALUATION OF HIGHLY ACTIVE TEST SUBSTANCES

Generally, growth inhibiting actions of test compounds were found to be bacteriostatic for cells exposed with 0.5×MIC and MIC of the test compounds and bactericidal at 2×MIC of the test compounds. When *Acinetobacter baumannii BDU-32* was treated with MIC of ethyl -2-(4- (2-ethoxy-2 -oxo ethyl) thiazole-2-yl) -6-hydroxy-6-methyl -3-oxo-4- (p- tolyl) -2,3,4,5,6,7- hexa hydro-1-H indazol-5-carboxylate, there was drop in number of living cells over the first 8 hours. After the 24 hours, number of viable cells was almost same as in the initial inoculum. This demonstrates the bacteriostatic effect of test compound against *Acinetobacter baumannii BDU-32* at MIC of the test substance. Death kinetics of *Acinetobacter baumannii BDU-32* treated with two times of the MIC of the test substance demonstrated consistent decrease in number of living cells over the period of experiment. After 24 hours, a decrease of greater than 3 log10 of the total colony forming units per mL in the initial sample was noted. This shows the bactericidal nature of test compound at two times of the MIC against *Acinetobacter baumannii BDU-32*. Time kill curves of diethyl 2,4-dicyano-3-(4-chloro phenyl)-8- morpholino -6-oxobicyclo[3.2.1]octane-2,4-

dicarboxylate against *Escherichia coli BDU-12* showed that number of living cells was slightly lower as compared to number of cells in the initial inoculum after 24 hours when treated with MIC of the test compounds. A decline of greater than 3 log10 of the colony forming units per mL in the original inoculum was recorded after 24 hours at 2×MIC of the test compound. This demonstrated the bactericidal effect of test compound at 2×MIC against *Escherichia coli BDU-12* (figure 6.1)

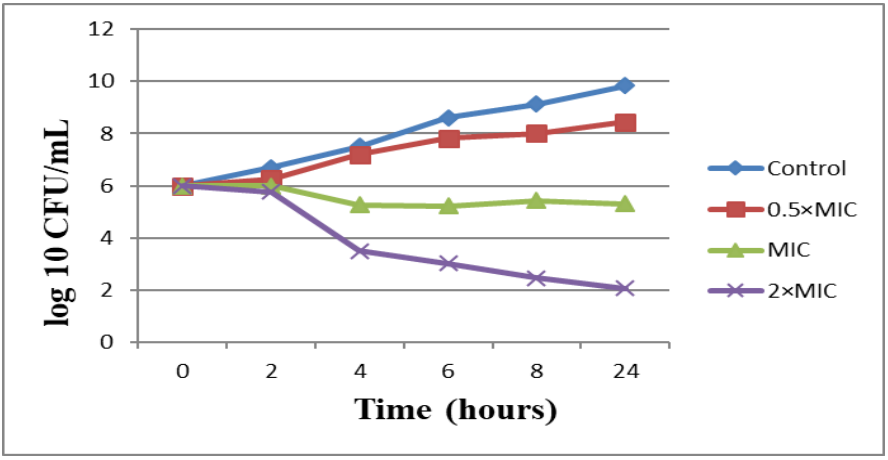

Figure 6.1. Death kinetics of *Escherichia coli BDU-12* when exposed to different concentrations of test compound

Incubation of *Klebsiella pneumoniae BDU-44*cells with a test compound concentration of 0.5×MIC (15.63 µg/ml) did not lead to a decrease in bacterial cell count. For *Klebsiella pneumoniae BDU-44* incubated with MIC of test compound (31.25µg/ml), the decrease in bacterial cell number was recorded until 8 hours. After 8 hours, the number of *Klebsiella pneumoniae BDU-44* remained constant until 24 hours. Test compound demonstrated bacteriostatic effect at MIC value against *Klebsiella pneumoniae BDU-44*. There was more than 4 log 10 decline in the living bacterial cells as compared to the number of the bacterial cells in the initial inoculum at 2×MIC of test compound. Time-kill kinetic studies indicated that test compound exhibited bactericidal actions when *Klebsiella pneumoniae BDU-44* was exposed to 2×MIC.

The time kill curves manifested that there was not any considerable reduction in number of viable cells when *Pseudomonas aeruginosa BDU-49*were exposed to 0.5×MIC of dimethyl 5-acetyl -1,3- dicyano -4- hydroxy -4- methyl -2,6- diphenyl cyclohexane-1,3-dicarboxylate. When *Pseudomonas aeruginosa BDU-49* was exposed MIC of the test compound, number of living cells was faintly elevated after 24 hours. This implies that the MIC of the test compound has bacteriostatic effect against *Pseudomonas aeruginosa BDU-49*. A decline of greater than 3 log10 of the total colony forming units per mL in the initial sample was recorded after 24 hours. This demonstrated that the bactericidal effect of test compound at 2×MIC against *Pseudomonas aeruginosa BDU-49*.

Incubation of *Staphylococcus aureus BDU-23* cells with concentration of 0.5×MIC (31.25 µg/ml) of 2-(1-acetyl-5-cyano-6-(2,4-dichlorophenyl)-2- methyl-4-oxo-3-aza bicyclo [3.1.0]hexan -2-yl) malononitrile led to a decrease in bacterial cell count for only first two hours (figure 6.2).

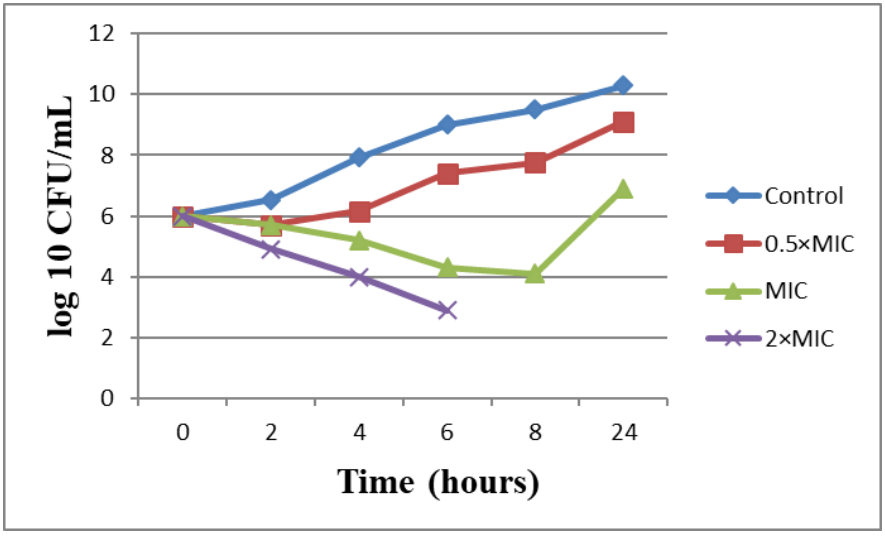

Figure 6.2. Death kinetics of *Staphylococcus aureus BDU-23*when exposed to different concentrations of test compound

Staphylococcus aureus BDU-23 incubated with MIC (62.5µg/ml) of compound, the decrease in bacterial cell number was recorded until 8 hours. Interestingly, after 8 hours the number of *Staphylococcus aureus BDU-23* soared until 24 hours

At the end, the number of the bacterial cells was a little higher as compared to the quantity of the bacterial cells in the initial inoculum. There was more than 3 log 10 decline in the number of the living bacterial as compared to the number of the bacterial cells in the initial inoculum, when *Staphylococcus aureus BDU-23* was exposed to two times of the MIC of test compound.

This demonstrated that viability of the *Staphylococcus aureus BDU-23* abolished within first six hours. Hence, 2-(1-acetyl-5-cyano-6-(2,4-dichlorophenyl)-2- methyl-4-oxo-3-aza bicyclo [3.1.0]hexan -2-yl) malononitrile was found to be has bacteriostatic against *Staphylococcus aureus BDU-23*the MIC and bactericidal at two times of the MIC.

DISCUSSION

Overall, functionally substituted cyclohexane derivatives showed variable antimicrobial profile against different types of bacterial and fungal pathogens. Bacterial test cultures were found to me more susceptible to test compounds as compared to fungal test cultures. Complex cell wall structure of the fungi (chitin) is thought to be responsible for less susceptibility of fungi. Test compounds manifested stronger antibacterial profile against Gram-negative bacteria than Gram-positive bacteria. This is due to the difference in structure of cell wall (amount of peptidoglycan) of Gram-negative bacteria and Gram-positive bacteria. Antimicrobial profile of these derivatives is found to be dependent on the position and types of different functional groups. *Acinetobacter baumannii BDU-32* was recorded to be the most sensitive test culture among all the Gram-negative bacteria while *Pseudomonas aeruginosa BDU-49* was the most resistant test culture among all the Gram-negative bacteria. *Staphylococcus aureus BDU-23* was the most resistant test culture among all the Gram-positive bacteria. *Bacillus mesentericus BDU-51* was observed to be the most susceptible among all the Gram-positive

bacteria. Our initial results of the agar well diffusion procedure were validated by minimum inhibitory concentrations of the test substances. Gram-negative bacteria were found to more susceptible (having lower minimum inhibitory concentration values) as compared to Gram-positive bacteria. Three concentrations of test compounds i.e. 0.5×MIC, MIC and 2×MIC were applied to test out the influence of test compounds on growth and reproduction of test cultures. Test compounds exhibited remarkable differences in growth curves of different test cultures as compared to growth curves without test compounds. Growth reduction effects were more significant for Gram-negative bacteria than Gram-positive bacteria. This implies that test derivatives were highly effective against Gram-negative bacteria than Gram-positive bacteria. Time kill curve assay to establish bactericidal or bacteriostatic effect of test compounds showed that antimicrobial effects of the test compounds were concentration dependent. Test compounds were found to be bacteriostatic at MIC and bactericidal at 2×MIC against susceptible test cultures.

MAIN RESULTS

1. Gram-negative bacteria were observed to be more vulnerable against test compounds than Gram-positive bacteria and yeast. All the compounds were effective against Gram-negative bacteria, while 32% of test substances were inactive against Gram-positive bacteria.
2. Monocyclic cyclohexane derivatives exhibited superior antimicrobial potential than spirocyclic cyclohexane derivative. This is due to difference in the nature and position of functional groups.
3. *Acinetobacter baumannii BDU-32* was the most sensitive test culture among Gram-negative bacteria, while *Staphylococcus aureus BDU-23* was the most sensitive test culture among Gram-positive bacteria.
4. Least minimum inhibitory concentration value was noted to be 31.25 µg/ml against *Acinetobacter baumannii BDU-32* by ethyl -2-(4-(2-ethoxy-2- oxo ethyl)thiazole -2-yl) -6-

hydroxy-6-methyl -3-oxo- 4- (p- tolyl) -2,3,4,5,6,7-hexa hydro-1H indazol-5-carboxylate and against *Klebsiella pneumoniae BDU-44* by ethyl-4- methyl-6-phenyl -2-oxocyclohex-3-en-1-carboxylate.

5. Turbidimetric measurements showed that functionally substituted cyclohexane compounds have significant potential to prevent the growth of test cultures at minimum inhibitory concentration values of test compounds.
6. Time kill assay demonstrated that test compounds had bacteriostatic effects at less than or equal to minimum inhibitory concentration (MIC) and bactericidal effects at greater than MIC value against different test cultures.
7. Ethyl -6- hydroxy- 6-methyl -3- oxo-4- phenyl-1,3,4,5,6,7-hexahydro benzo [c] isooxazole-5- carboxylate showed high antimicrobial profile only against all the Gram-negative bacteria, Thus, this compound can be used as selective antimicrobial agent against Gram-negative bacteria (Patent No. İ 2020 0097).

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The defense will be held on **“10” September 2021** at **14-00** at the meeting of the Dissertation council FD 1.07 operating at Institute of Microbiology of ANAS.

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