INHIBITION OF BIOFILM PRODUCING GRAM NEGATIVE CLINICAL ISOLATES AND THEIR ANTIBIOGRAM PATTERN

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Abstract:

Background: Bacterial biofilm is a major virulence factor that posses a threat to patients leading to chronic infections. Therefore, it is crucial to identify biofilm production as well as their inhibition and reduction. This study was an attempt to investigate biofilm production among gram-negative isolates and assessment of inhibitory and reduction potential of EDTA and DMSO towards them and also observe the antimicrobial resistance pattern among biofilm producers and biofilm non-producer.

Methods: Isolation and identification of bacterial isolates were performed by standard microbiological methodology. The antibiotic susceptibility pattern was determined by the Kirby Bauer disk diffusion method and β-lactamases by the combination disk method. Biofilm formation was detected through Tissue Culture Plate(TCP) method, and different concentrations of EDTA and DMSO were used to determine their inhibitory and reduction property against biofilm. Both inhibition and reduction by the various concentration of EDTA and DMSO were analyzed using paired t-test.

Results: Among the 110 clinical isolates 61.8% were found to be Multidrug resistance(MDR) with the 33 (30%) produced Extended-spectrum β-lactamases(ESBL), 16 (14.5%)Metalloβ-lactamases(MBL) and 9 (8%)Klebsiella pneumonia carbapenemase(KPC). Biofilm formation was detected in 35.4% of isolates. Biofilm producing organisms showed antibiotics resistance to Cephalosporins, Chloramphenicol, Gentamycin, and Carbapenem. The inhibition and reduction of biofilm were significantly lower ($p<0.05$) for 1mM of EDTA and 2% of DMSO.

Conclusions: EDTA and DMSO were found to possess potential activity against biofilm. Hence, EDTA and DMSO might be used invitro as an effective antibiofilm agent to control the biofilm-associated infection and for a possible therapeutic approach.

Keywords: Biofilm formation, Biofilm inhibition, Gram Negative bacteria, Antibiogram

What's known?

EDTA possesses a potent activity for inhibition and reduction of biofilm. According to the study of Yahya et al. 32% of DMSO was used for the reduction of biofilm of *Escherichia coli* and *Pseudomonas aeruginosa.*

What's new?

In this study different concentration of DMSO was used for to access the inhibition and reduction of biofilm of various gram negative organisms (*Escherichia coli, Klebsiella* species*,* *Pseudomonas aeruginosa* and *Acinetobacter* species).

Introduction

Bacterial biofilm is the community of microbial cells that adhere to the solid surface, which can either be biotic or abiotic and remains enclosed in a self-produced polymeric matrix or slime(1). Depending on bacterial species, strain type and environmental conditions, the biofilm matrix consists of substances of diverse chemical nature such as exopolysaccharides, proteins, and extracellular DNA (eDNA)(2). Biofilm formation evades the host immune response, conventional antimicrobial agents, biocides through the "bulky-shields" built by extracellular polymeric substances(EPS)(3). The biofilm matrix acts as a barrier for diffusion and fails to penetrate the antimicrobial agent;consequently, it results in differentiate into persister cells and

inactivate the action of antibiotics(4, 5). Biofilms are mainly associated with tissue or indwelling medical devices such as implants and catheters and lead to chronic or recurrent infections(6). According to the CDC and NIH, 65 to 80 percent of all persistent infection is triggered by the organism that creates biofilm, lead therapeutic failure(7). Biofilm-associated pathogens possess higher resistance, i.e. 10-1000 times to antibiotic treatment and 150-3000 times to disinfectants compared to planktonic cells, including host defense mechanisms leading to difficulty in elimination of established biofilm infections(6).

An alternative to traditional antibiotic treatment, new antibiofilm agents, have been introduced. Currently, most of the research is focused on the development of non-toxic antibiofilm agents, as such molecules may not lead to future drug resistance(8). DMSO and EDTA are used in this study to determine their activity for the prevention and removal of biofilm. EDTA inhibits gram-negative bacteria by its metal chelation property, as it chelates the cation, which was responsible for stabilizing the negatively charged polysaccharides(9). Besides, DMSO solubilized the EPS matrix by the formation of electrostatic repulsion due to which destabilization of biofilm occurs (10).Therefore, the objective of this analysis was the identification of biofilm growing as well as its inhibition and reduction by EDTA and DMSO.This study also aimed to investigate the antimicrobial resistance pattern among biofilm-producing and biofilm non-producing organisms in clinical gram-negative isolates.

METHODS

This laboratory-based cross-sectional study was conducted in Manmohan Memorial Institute of Health Sciences from February 2018to July 2018 (for six months), which included Gram-Negative isolates from various clinical samples like urine, sputum, blood, pus and sterile fluids.

Samples were cultured in Blood agar (HiMedia, India) and Mac Conkey agar(HiMedia, India). In contrast, chocolate agar (HiMedia, India) was also used for respiratory specimens and incubated at 37°C for 24 hours. The identification of significant isolates was performed based on standard microbiological techniques, which involved the morphological appearance of the colony, gram's staining reactions, oxidase test, and other biochemical properties. The purity plate was employed to ensure that the inoculation used for the biochemical test is a pure culture.

Antibiotic Susceptibility testing

The antibiotic susceptibility profile of the pathogens towards different antibiotics was determined by Kirby Bauer disk diffusion method using Muller Hinton agar (MHA)(HiMedia, India)as per the Clinical and Laboratory Standards Institute(CLSI)(11). The following antibiotics discs were used; Amoxycillin (12µg), Cefixime (5µg), Cefotaxime (30µg), Ceftazidime (30µg), Chloramphenicol (30µg), Ciprofloxacin (5µg), Cotrimoxazole (25µg), Gentamycin (10 µg), Imipenem (10 µg), Levofloxacin (5 µg), Meropenem (10 µg), Piperacillin-Tazobactam (100/10 µg), Tetracycline (30 µg), Tigecycline (15 µg), Polymyxin B (300 units) and Colistin Sulphate (10 µg). The isolate resistant to at least one antimicrobial agent of three classes of the antimicrobial agent was regarded as MDR(12). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 was also tested, in every set of experiment, in parallel, as quality control.

Detection of ESBL,Metallo Beta Lactamases(MBL)and *Klebsiella pneumonia* carbapenemase(KPC) β-lactamases

Testing for ESBL producer

For the detection of ESBL production, a screening test was carried out by using Ceftazidime (CAZ) (30ug) and Cefotaxime (CTX) (30ug) discs (HI Media India). If the zone of inhibition (ZOI) was equal to or less than 22mm for Ceftazidime and equal or less than 27mm Cefotaxime, the isolate was considered as a potential ESBL producer as recommended by CLSI. Further confirmation of ESBL production was carried out by a combined disk test (CDT). In this method, Cefotaxime (30ug) alone and Cefotaxime in combination with Clavulanic acid (CA) (30ug/10 µg) wereplaced 20 mm away on test strain inoculated MHA plate. An increase ZOI of >5mm for a combined disc in comparison to Ceftazidime alone confirmed ESBL production(11)

Testing for MBL and KPC

Screening of both MBL and KPC were done using the disk Meropenem (MRP) and Imipenem (IPM). The isolates that were non-susceptible to Imipenem were considered presumptive MBL producers.

In this method, test isolates (comparable to 0.5Mc Farland) were inoculated in MHA plate where two IPM discs were placed 25mm away from the center, one with 10µl of 0.1M (292 µg) anhydrous Ethylene diamine tetraacetic acid(EDTA) (Sigma Aldrich) and one IPM alone. The inhibition zone of the EDTA+ Imipenem and Imipenem alone was compared. The zone of inhibition of IPM+EDTA is > 4mm; that of IPM alone is considered MBL production(13).

Similarly, for KPC detection, two MRP (10 µg) discs are placed 20mm away from the center, one with 20µl of 3-Amino phenylboronic acid (3-APBA) containing 400 µg and one MRP alone. The inhibition zone of the APBA+ Meropenem and Meropenem alone was compared. Zone of inhibition of MRP+APBA is ≥5mm that of MRP alone is considered KPC production(14)

Detection of Biofilm Production

Biofilm production by clinical isolates was carried out by the tissue culture plate technique. Organisms isolated from fresh agar plates were inoculated in 2ml of Luria Bertani broth (HiMedia, India) with 2% glucose and incubated at 37°C for 24 hours. Then cultures were diluted at the ratio of 1:100 with a fresh medium. 200µl of the diluted culture of different strains were inoculated in each well of sterile flat bottom 96 well polystyrene tissue culture plates and incubated for 24 hours at 37°C

After incubation, contents of each well removed and washed with 0.2mL of phosphate buffer saline (pH 7.2) three times. Then, the formed biofilm by bacteria which were adherent to the wells were fixed by keeping at 60°C for 1 hour and were stained by crystal violet (2%). Excess stain was removed by using deionized water by rinsing three times and subsequently decolorized with 30% acetic acid. Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA autoreader at wavelength 570nm.

Uninoculated wells containing broth were considered as a negative control. The experiment was performed in triplicate for two times. The average optical density(OD) values of each test strain and negative control were calculated, and final OD values of a test strain were expressed as average OD value of the strain reduced by OD cut-off value (ODc) of the negative control. The interpretation of biofilm production was done according to Stepanovic et al. criteria. ODc had been specified as three standard deviations (SDs) above the negative control(15-17).

Inhibition of biofilm

In the assessment of EDTA and DMSO's capacity to inhibit the production of biofilms, organisms were separately grown overnight in LB broth with 2% glucose. Then an equal volume of the culture and various concentrations of inhibiting agents were transferred into sterile 96-well polystyrene tissue culture plates. For about 24 hours, the plates were incubated at 37°C and

washed three times with 200µl of sterile PBS, cleaned and stained with 2% crystal violet.The residual stain removed by rinsing with purified water and decolorized with 30% acetic acid. Optical density (OD) of stained adherent biofilm was obtained by using a micro ELISA auto reader at a wavelength of 570nm. Wells containing LB broth was used as a negative control(15, 16, 18). Inhibition data were presented in the form of magnitude.

Magnitude of inhibition= OD before inhibition of biofilm divided by OD after treatment with inhibiting agent

Reduction of biofilm

It was performed to evaluate the ability of the compound, i.e. EDTA and DMSO, to dissociate gram-negative biofilm. 200µL of each bacterial suspension was inoculated in sterile 96-well polystyrene tissue culture plates and were further incubated for 24 hours at 37°C for biofilm production without agitation. The formed biofilm was then exposed for next 24 hours with different concentrations of inhibiting compounds by adding it to the microtiter plate.After that, the wells washed three times with 200µL of sterile PBS, dried and stained with 2% crystal violet for ELISA reading. After rinsing, the stain was decolorized by 30% acetic acid, and the absorbance of the adherent biofilm was measured at 570 nm in a microplate reader(15, 16, 18). Reduction data were presented in the form of magnitude.

Magnitude of reduction= OD before reduction of biofilm divided by OD after treatment with reducing agent

Statistical Analysis

Findings were manually recorded and entered into the database. The analysis was done by SPSS Version 20 (IBM corporation, Armonk, NY, USA). The students paired t-test used to evaluate

179 the mean difference between the OD value for control (without an inhibiting agent) and different
180 concentrations of EDTA and DMSO used for both inhibition and reduction.

181 **RESULT**

182 During the research period, a total of 110 non-replicative gram-negative clinical organisms were
183 isolated.Among which 68 were from urine, 38 were from sputum, 17 were from pus and wound
184 swabs, 13 were from blood, and 2 were from bile.*Escherichia coli* was predominant pathogen 51
185 (36.9%) followed by *Klebsiella* species 32 (23.18%) (Table1).

186 **Antimicrobial resistance**

187 **Antibiogram of *Enterobacteriaceae***

188 In our study, organisms showed variable responses towards different antibiotics tested. Among
189 *Enterobacteriaceae, Escherichia coli* were highly resistance against Amoxyciilin (92.2%),
190 followed by Cotrimoxazole (64.7%), *Klebsiella* spp., showed high resistance to third-generation
191 Cephalosporins (84.45%), Ciprofloxacin (71.9%) and Cotrimoxazole (71.9%). The resistance
192 rate of tested antibiotics towards *Klebsiella* spp. is higher than *Escherichia coli* (Table2).
193 A*cinetobacter calcoaceticus baumannii* complex have demonstrated higher tolerance to
194 antimicrobial agents among non-fermenters. All isolates were complete resistant to Piperacillin-
195 Tazobactam, Cephalosporin, Ciprofloxacin, and Gentamycin and 88.9% to carbapenems whereas
196 the rate was lower for *Pseudomonas*, 44.4% to Ceftazidime, 38.9% to Piperacillin-Tazobactam
197 and Carbapenem were effective against *Pseudomonas* (22.2% resistance)(Table3).

198 **Incidence of MDR and Beta-lactamases production**

199 Out of 110 gram-negative isolates, 68 (61.8%) were found multidrug resistance (MDR). Entire
200 isolates of *Acinetobacter calcoaceticus baumannii* complex and 75% of *Klebsiella* were found
201 to be MDR. The rate of ESBL production was detected in 30% of isolates. Likewise, 14.5% and

8.1% of isolates were MBL and KPC producers, respectively. *Klebsiella* species was major ESBL producer 15/32 (46.9%), followed by *Escherichia coli* 18/51 (35.3%). Similarly, major MBL producers were *Acinetobacter calcoaceticus baumannii* complex 6/9(66.7%), Klebsiella spp(8/32) (25%). The prevalence of KPC producers was found to be 3/9 (33.3%)in*Acinoetobacter calcoaceticus baumannii* complex, and 4/32(12.5%) in *Klebsiella* species (Table4).

Frequency of Biofilm formation

TCP method detected 39 (35.4%) biofilm producers of which *Pseudomonas aeruginosa* 16/18 (88.9%), *Acinetobacter calcoaceticus baumannii* complex7/9(77.8%), *Klebsiella*9/32(28.1%) and *Escherichia coli*7/51(13.7%).Strong biofilm production showed by *Pseudomonas aeruginosa*(2/18)and*Acinetobacter calcoaceticus baumannii* complex (2/9) (Table5).

Comparison of antibiotics resistance pattern among biofilm producer and biofilm non-producer

The association of antimicrobial resistance is higher with biofilm producers. Biofilm producing isolates were resistant to antibiotics such as the Cephalosporin, Chloramphenicol, Gentamycin, Piperacillin/Tazobactam, and Carbapenems compared to biofilm non-producer (Table6).

Comparison of MDR pattern and β-lactamases among biofilm producer and non-producer

In this study, among biofilm producers, 25(64.1%) isolates were found to be MDR, 8(20.5%) were ESBLproducer followed by 10(25.6%) were MBL, and 6(15.4%) were KPC producer.

Biofilm inhibition and reduction

Different concentrations of EDTA in millimole (mM)(0.5, 1, 2, 4, and 5mM)have been analyzed for their effects on inhibition and biofilm reduction. Similarly, we used different concentration of

DMSO 1%, 2%, 4%, 8%, 16% and 24%. As seen by biofilm quantification using crystal violet at 570nm, EDTA and DMSO both lead to the inhibition and reduction of biofilm in a dose-dependent manner. They are also species-specific (Figure1 and 2).

Values from each treatment category were compared to untreated biofilm control using a t-test, and the outcome for inhibition and reduction in all isolates was significantly lower (p-value < 0.05) from 1mM EDTA and 4% DMSO.

DISCUSSION

Biofilm leads to the spread of antimicrobial resistance and generation of more virulent strain as it favors horizontal gene transfer by which resistance and virulent factor may pass among bacteria(19). The ineffectiveness of oral antimicrobial agents in eradicating the bacterial cells in biofilm had led off the search for topical therapies. The development of novel agents that prevent or eliminate biofilm without involving in the resistance mechanism is needed for a potential therapeutic approach to control the infections associated (20). In this study, we examine the potent use of EDTA and DMSO as biofilm inhibiting and reducing agents against various gram-negative isolates. We also observe the distribution of antimicrobial susceptibility patterns beta-lactamases among biofilm producer and non-producer.

In our findings, 61.8% of gram-negative isolates were MDR. The highest being in *Acinetobacter calcoaceticus baumannii* complex (100%), *Klebsiella* species (75%), *Escherichia coli*(56.8%), and *Pseudomonas aeruginosa* (33.3%).This result was similar to the study by Fatima et al. and Domenico et al. (21,22). The increasing prevalence may be due to the acquisition of various drug resistance mechanisms such as beta-lactamases enzymes, efflux pumps, biofilm formation, and decreased drug uptake. It raises the need for the daily investigation of possible MDR strains(23, 24)

Resistance to antibiotics exacerbated as the knowledge towards the organism's susceptibility to antimicrobial agents is vital for treatment regimens. Among *Enterobacteriaceae,* 92.2% *Escherichia coli* were resistant against Amoxicillin, 84.4% of *Klebsiella* spp. were resistant against third-generation cephalosporin. In the case of *Escherichia coli*, a study performed at Tanzania was harmonical (25). In contrast to our study Eldomany et al. reported that resistance towards fluoroquinolones, aminoglycosides and Carbapenem was lower in *Klebsiella* spp.(26)**.** Increasing resistance rate to commonly used antibiotics is mainly due to improper use, easy access, and inadequate monitoring, which causes disadvantages for the health care system as it can significantly affect patients management(24, 27).

Among non-fermenter, *Acinetobacter calcoaceticus baumannii* were found more resistance to antimicrobial agents. All isolates (n=9) were utterly resistant to Piperacillin-Tazobactam, Cephalosporin, Ciprofloxacin, and Gentamycin and 88.9% to Carbapenems which found similar with the study of Parajuli et al. from Nepal(28). However, our study was nearly twofold higher than that reported by Mishra et al.(29).

Beta-lactam is the drug of choice for the treatment of infections caused by gram-negative organisms(30). Out of total gram-negative isolates, 30.0% were found to be ESBL producer of which highest rate in *Klebsiella* species 46.9% that was similar to the previous studies (31,32).

Among the isolates, 14.5% were found to produce MBL of which a higher rate in *Acinetobacter calcoaceticus baumannii* complex (66.7%) followed by *Klebsiella* species (25%).Our study was harmonical with the study of Chaudhary et al.(33) from Nepal reported 13.6% MBL producer. The increased prevalence of MBL rates may be due to different geographical areas and a gradual rise in the use of carbapenems(34).

The KPC-producing isolates are rising and are involved in life-threatening nosocomial and systemic infections causing limited therapeutic options(35). In our study, *Acinetobacter calcoaceticus baumannii* complex and *Klebsiella* species were major KPC producer.The study by Robledo et al. reported 11.79% as KPC producer among which higher rate in *Acinetobacter calcoaceticus baumannii*c omplex followed by *Klebsiella pneumoniae*. The difference in KPC rates may be due to the misuse of antibiotics, extensive use of Carbapenem due to an increased number of ESBL positive isolates, and the horizontal transmission of KPC genes (36).

Biofilm related infections are more troublesome and are expensive to treat(7). Among 110 gram-negative clinical isolates, the incidence of biofilm producer was found to be 39(35.4%), of which 5(12.8%) were strong, 7(17.94%) moderate, and 27(69.2%) were weak biofilm producer. In this study, *Pseudomonas aeruginosa* was found to be a potent biofilm producer followed by *Acinetobacter calcoaceticus baumannii* complex, *Klebsiella* species and *Escherichia coli* was detected least producer. A study performed by Hassan et al. from Pakistan showed 37% of gram-negative isolates as biofilm positive(37).In the study by Sanchez JR et al., 57.7% gram-negative isolates showed biofilm production with the highest rate in *Pseudomonas aeruginosa*, followed by *Klebsiella pneumoniae*, *Acinetobacter calcoaceticus baumannii* complex, and *Escherichia coli* (38).

Biofilm-producing organisms exhibited a high degree of antimicrobial resistance compared to biofilm non-producer against cefixime, cefotaxime, Ceftazidime, chloramphenicol, gentamycin, piperacillin/tazobactam, carbapenems, and Tigecycline. Carbapenems and Tigecycline were the most effective drugs against biofilm producers. Similarly, the study of Mishra et al. (16), Panda et al.(39) and Zubair et al.(40) reported antimicrobial resistance exhibited by biofilm producer was higher than biofilm non-producer. The higher resistance could be due to the close contact of

293 organisms in biofilm, activity of exopolysaccharide matrix, growth rate alteration, pH, and
294 osmotic variation, resistant gene or plasmid transfer among isolates within a biofilm (41).
295 On the comparative evaluation of drug resistance pattern and biofilm production among gram-
296 negative isolates, it was observed that 66.1% of biofilm producers were MDR which was similar
297 with the study of Aasti et al. (42) and Fatima et al.(21). In this study, the rate of production of
298 ESBL among biofilm producers was far lower than the study by Neupane et al.(43) and Dhakal
299 et al.(44). Similarly, among biofilm producers, the rate of MBL production was 25.6%, which
300 was lower than the study bySinghai et al. (45). Likewise, KPC production was seen in 15.4%
301 among biofilm producers, which was similar to the study of Hussein et al. (46). The combination
302 of virulence factors such as biofilm and various enzymes production might be species-specific,
303 and the concurrent detection of these factors helps in designing new therapies thatare effective
304 towards them (45).
305 Resistance to various antibiotics, mainly due to biofilm formation, is on the rise. Biofilm
306 producing microorganisms causes multiple infections in foreign devices leading to a severe
307 complication resulting in high morbidity, mortality, medical costs, and hospital stay. So, there is
308 a critical need for identifying therapeutic strategies that are directed toward the inhibition of
309 biofilm formation and the effective treatment of biofilms once they have formed (47). In our
310 study, we have used different concentrations of EDTA and DMSO as biofilm control agents.
311 0.5mM,1mM,2Mm, 4mM, and 5mM EDTA were used, which has the activity towards the
312 biofilm. Among them, 5mM was most effective. The inhibitory effect of EDTA was higher for
313 all isolates as compared to the biofilm reduction capacity. The action of EDTA was
314 concentration-dependent and species-specific with the highest inhibition of *Acinetobacter*

biofilm 3.37 (70%) and a more significant reduction *in Pseudomonas* biofilm magnitude 2.5 (60.7%).

The inhibitory effect of EDTA on biofilm formation of *Escherichia coli* was 2.26 times (55%) and reduction activity (39.4%) which was lower than the study of Gawad et al. (48) and Chaudhary et al. (49). In a study of Bakri et al. 8mg/ml (21mM) of EDTA after 1 hr exposure, the percentage reduction in the viable count of established biofilm of *Pseudomonas aeruginosa* by 98.98% and *Escherichia coli* by 53.18% which was similar to our study showing the higher activity of EDTA towards *Pseudomonas aeruginosa* as compared to *Escherichia coli* (9).

Furthermore, various DMSO concentrations were used as the antibiofilm agent. The increasing concentration showed a better effect in biofilm inhibition with the highest magnitude *Acinetobacter*3.70, *Pseudomonas*3.0, *Klebsiella* 2.29, and *Escherichia coli* 2.23. However, the reduction rate was lower as compared to the inhibitory effect having a magnitude between 1 to 2 for all isolates. The magnitude of inhibition by 2% DMSO against *Pseudomonas aeruginosa* biofilm was similar to the study of Guo et al.(50). The reduction activity of 24% DMSO for *Escherichia coli* showed a magnitude of 1.69(40.8%) and in the case of *Pseudomonas aeruginosa* (42.8%)while the study of Yahya et al. reported 38.6% reduction in *E. coli* and 60.7% in *Pseudomonas aeruginosa* by 32% DMSO. However, in his study, the concentration of DMSO was high 32% (10).

However, this study has some limitations. Our study was based on the phenotypic method of biofilm detection. This study only performed the susceptibility pattern with the commercially available antibiotic concentration, while it does not provide information on the minimum inhibitory concentration of any particular antibiotics. Our study suggests that the large sample size may be taken into account to establish the inhibition and reductionof biofilm production.

Conclusion:

The study demonstrated a high level of biofilm production among gram-negative isolates. Consequently, a higher rate of antimicrobial resistance was present in biofilm producers in comparison to biofilm non-producers. EDTA and DMSO as non-antibiotic agents were found to possess potential activity against biofilm. EDTA and DMSO had significantly inhibited and reduced the biofilm formation in a dose-dependent manner and are also species-specific. Thus, our study recommendsthat EDTA and DMSO can be potentially useful for biofilm-related infections. However, to evaluate the efficacy of EDTA and DMSO, further experiments are needed. These finding would be beneficial in the future to battle against biofilm producer gram-negative microorganisms.

Abbreviation

ASM: American Society for Microbiology

CLSI: Clinical and Laboratory Standard Institute

NIH: National Institute of Health

CDC: Centers for Disease Control and Prevention

WHO: World Health Organization

GNB: Gram Negative Bacilli

ICU: Intensive Care Unit

EDTA: Ethylene diamine tetra acetic acid

DMSO: Dimethyl Sulphoxide

CDT: Combined Disk Test

MDR: Multi Drug Resistant

ESBL: Extended Spectrum Beta Lactamases

MBL: Metallo Beta Lactamases

KPC: *Klebsiella pneumoniae* carbapenemase

TCP: Tissue Culture Plate

Spp: Species

MMIHS: Manmohan Memorial Institute of Health Sciences

ELISA: Enzyme linked immunosorbent assay

SPSS: Statistical Package for Social Science

Acb: *Acinetobacter calcoaceticus baumannii*

Ethical Approval

This research was approved by the Institutional Review Committee of Manmohan Memorial Institute of Health Sciences, Kathmandu, Nepal. Letter of approval (MMIHS-IRC-225) was obtained after submitting and presenting the proposal to the committee.

Author contribution

OS, GJ and PRK designed the study. OS, NS, SK and SM conducted the laboratory tests. OS, TBT,SP collected and analyzed the data. OS prepared the manuscript. OS, GJ and SP edited and reviewed the manuscript. All the authors critically reviewed and revised the manuscript draft, and approved the final version of manuscript.

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References

1. CR M. Diagnostic Microbiology. New Delhi: Elsevier; 2007.

2. Sanchez LM, Cheng AT, Warner CJ, Townsley L, Peach KC, Navarro G, et al. Biofilm formation and detachment in gram-negative pathogens is modulated by select bile acids. PloS one. 2016;11(3):e0149603.

3. Nakao R, Ramstedt M, Wai SN, Uhlin BE. Enhanced biofilm formation by Escherichia coli LPS mutants defective in Hep biosynthesis. PloS one. 2012;7(12):e51241.

4. Paraje M. Antimicrobial resistance in biofilms. Science against microbial pathogens: communicating current research and technological advances. 2011:736-44.

5. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clinical microbiology reviews. 2002;15(2):167-93.

6. Jefferson KK. What drives bacteria to produce a biofilm? FEMS Microbiology Letters. 2004;236(2):163-73.

7. Lebeaux D, Chauhan A, Rendueles O, Beloin C. From in vitro to in vivo models of bacterial biofilm-related infections. Pathogens. 2013;2(2):288-356.

399 8. Rabin N, Zheng Y, Opoku-Temeng C, Du Y, Bonsu E, Sintim HO. Agents that inhibit
400 bacterial biofilm formation. Future medicinal chemistry. 2015;7(5):647-71.
401 9. Al−Bakri A, Othman G, Bustanji Y. The assessment of the antibacterial and antifungal
402 activities of aspirin, EDTA and aspirin−EDTA combination and their effectiveness as antibiofilm
403 agents. Journal of applied microbiology. 2009;107(1):280-6.
404 10. Yahya MFZR, Alias Z, Karsani SA. Antibiofilm activity and mode of action of DMSO alone
405 and its combination with afatinib against Gram-negative pathogens. Folia microbiologica.
406 2018;63(1):23-30.
407 11. Watts JL, Shryock T, Apley M, Brown SD, Gray JT, Heine H, et al. Performance standards
408 for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals;
409 approved standard—third edition. 2008.
410 12. Magiorakos AP, Srinivasan A, Carey R, Carmeli Y, Falagas M, Giske C, et al. Multidrug−
411 resistant, extensively drug−resistant and pandrug−resistant bacteria: an international expert
412 proposal for interim standard definitions for acquired resistance. Clinical microbiology and
413 infection. 2012;18(3):268-81.
414 13. Franklin C, Liolios L, Peleg AY. Phenotypic detection of carbapenem-susceptible metallo-
415 β-lactamase-producing gram-negative bacilli in the clinical laboratory. Journal of clinical
416 microbiology. 2006;44(9):3139-44.

14. Tsakris A, Poulou A, Pournaras S, Voulgari E, Vrioni G, Themeli-Digalaki K, et al. A simple phenotypic method for the differentiation of metallo-β-lactamases and class A KPC carbapenemases in Enterobacteriaceae clinical isolates. Journal of antimicrobial chemotherapy. 2010;65(8):1664-71.

15. Jain S, SenGupta M, SarKar S, GhoSh S, Nandi A, Sinha A, et al. Can EDTA Change MRSA into MSSA? A Future Prospective! Journal of clinical and diagnostic research: JCDR. 2016;10(2):DC22.

16. Mishra SK, Basukala P, Basukala O, Parajuli K, Pokhrel BM, Rijal BP. Detection of biofilm production and antibiotic resistance pattern in clinical isolates from indwelling medical devices. Current microbiology. 2015;70(1):128-34.

17. Kırmusaoğlu S. The Methods for Detection of Biofilm and Screening Antibiofilm Activity of Agents. Exopolysaccharides-Methods of Preparation and Application: IntechOpen; 2019.

18. Costa GA, Rossatto FC, Medeiros AW, Correa APF, Brandelli A, Frazzon APG, et al. Evaluation antibacterial and antibiofilm activity of the antimicrobial peptide P34 against Staphylococcus aureus and Enterococcus faecalis. Anais da Academia Brasileira de Ciências. 2018;90(1):73-84.

19. Costerton J, Montanaro L, Arciola CR. Biofilm in implant infections: its production and regulation. The International journal of artificial organs. 2005;28(11):1062-8.

20. Alandejani T, Marsan J, Ferris W, Slinger R, Chan F. Effectiveness of honey on Staphylococcus aureus and Pseudomonas aeruginosa biofilms. Otolaryngology—Head and Neck Surgery. 2009;141(1):114-8.

21. Fatima S, Prasanthi K, Nagamani K. Comparative evaluation of biofilm production in multidrug resistant and sensitive Gram negative clinical isolates. Int J Curr Microbiol App Sci. 2015;4:918-26.

22. Mammina C, Di Carlo P, Cipolla D, Giuffre M, Casuccio A, Di Gaetano V, et al. Surveillance of multidrug-resistant gram-negative bacilli in a neonatal intensive care unit: prominent role of cross transmission. American journal of infection control. 2007;35(4):222-30.

23. Tenover FC. Mechanisms of antimicrobial resistance in bacteria. American journal of infection control. 2006;34(5):S3-S10.

24. Parajuli NP, Maharjan P, Joshi G, Khanal PR. Emerging perils of extended spectrum β-lactamase producing enterobacteriaceae clinical isolates in a teaching hospital of Nepal. BioMed research international. 2016;2016.

25. Mshana SE, Kamugisha E, Mirambo M, Chakraborty T, Lyamuya EF. Prevalence of multiresistant gram-negative organisms in a tertiary hospital in Mwanza, Tanzania. BMC research notes. 2009;2(1):49.

26. Eldomany R, Abdelaziz NA. Characterization and antimicrobial susceptibility of gram negative bacteria isolated from cancer patients on chemotherapy in Egypt. Archives of Clinical Microbiology. 2011;2(6).

27. Pradhan B, Pradhan S. Prevalence of Urinary Tract Infection and Antibiotic Susceptibility Pattern to Urinary Pathogens in Kathmandu Medical College and Teaching Hospital, Duwakot. Birat Journal of Health Sciences. 2017;2(1):134-7.

28. Parajuli NP, Acharya SP, Mishra SK, Parajuli K, Rijal BP, Pokhrel BM. High burden of antimicrobial resistance among gram negative bacteria causing healthcare associated infections in a critical care unit of Nepal. Antimicrobial Resistance & Infection Control. 2017;6(1):67.

29. Mishra SK, Rijal BP, Pokhrel BM. Emerging threat of multidrug resistant bugs–Acinetobacter calcoaceticus baumannii complex and methicillin resistant Staphylococcus aureus. BMC research notes. 2013;6(1):98.

30. Heesemann J. Mechanisms of resistance to beta-lactam antibiotics. Infection. 1993;21:S4-9.

31. Doddaiah V, Anjaneya D. Prevalence of ESBL, AmpC and carbapenemase among Gram negative bacilli isolated from clinical specimens. Am J Life Sci. 2014;2(2):76-81.

32. Khanal S, Joshi DR, Bhatta DR, Devkota U, Pokhrel BM. β-lactamase-producing multidrug-resistant bacterial pathogens from tracheal aspirates of intensive care unit patients at National Institute of Neurological and Allied Sciences, Nepal. ISRN microbiology. 2013;2013.

33. AK C. Metallo-Beta-Lactamase Producing Gram-Negative Bacteria among Patients Visiting Shahid Gangalal National Heart Centre. Austin Journal of Microbiology. 2016;2(1).

34. Bora A, Sanjana R, Jha BK, Mahaseth SN, Pokharel K. Incidence of metallo-beta-lactamase producing clinical isolates of Escherichia coli and Klebsiella pneumoniae in central Nepal. BMC research notes. 2014;7(1):557.

35. Nordmann P, Cuzon G, Naas T. The real threat of Klebsiella pneumoniae carbapenemase-producing bacteria. The Lancet infectious diseases. 2009;9(4):228-36.

36. Robledo IE, Aquino EE, Vázquez GJ. Detection of KPC gene in Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Acinetobacter baumannii during a PCR-based nosocomial surveillance study in Puerto Rico. Antimicrobial agents and chemotherapy. 2011.

37. Hassan A, Usman J, Kaleem F, Omair M, Khalid A, Iqbal M. Detection and antibiotic susceptibility pattern of biofilm producing Gram positive and Gram negative bacteria isolated from a tertiary care hospital of Pakistan. Malays J Microbiol. 2011;7(1):57-60.

485 38. Sánchez CJ, Mende K, Beckius ML, Akers KS, Romano DR, Wenke JC, et al. Biofilm
486 formation by clinical isolates and the implications in chronic infections. BMC infectious diseases.
487 2013;13(1):47.
488 39. Panda PS, Chaudhary U, Dube SK. Study of biofilm production and antimicrobial
489 sensitivity pattern of uropathogens in a tertiary care hospital in North India. International
490 Journal Of Community Medicine And Public Health. 2016;3(9):2421-6.
491 40. Zubair M, Malik A, Ahmad J, Rizvi M, Farooqui KJ, Rizvi MW. A study of biofilm
492 production by gram-negative organisms isolated from diabetic foot ulcer patients. Biol Med.
493 2011;3(2):147-57.
494 41. Shashikala V. Diabetic foot ulcers and biofilm formation-The culprits.
495 42. Asati S, Chaudhary U. Prevalence of biofilm producing aerobic bacterial isolates in burn
496 wound infections at a tertiary care hospital in northern India. Annals of burns and fire disasters.
497 2017;30(1):39.
498 43. Neupane S, Pant ND, Khatiwada S, Chaudhary R, Banjara MR. Correlation between
499 biofilm formation and resistance toward different commonly used antibiotics along with
500 extended spectrum beta lactamase production in uropathogenic Escherichia coli isolated from
501 the patients suspected of urinary tract infections visiting Shree Birendra Hospital, Chhauni,
502 Kathmandu, Nepal. Antimicrobial resistance and infection control. 2016;5(1):5.

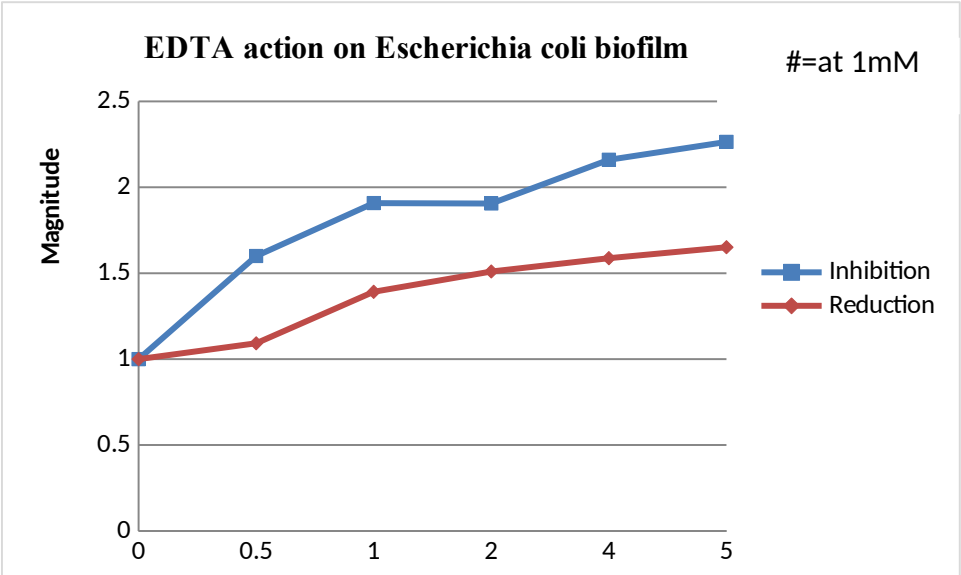
503 44. B D, LB, UM, SS, S aT. BIOFILM PRODUCTION AMONG EXTENDED SPECTRUM B-
504 LACTAMASE
505 PRODUCING AND NON-PRODUCING ESCHERICHIA COLI ISOLATES. GoldenGate Journal of
506 Science & Technology 2017;3:63-7.
507 45. Singhai M, Rawat V, Goyal R. Concomitant detection of biofilm and metallo-beta-
508 lactamases production in gram-negative bacilli. Indian Journal of Pathology and Microbiology.
509 2013;56(3):276.
510 46. Nadheema Hammood Hussein SNA-A, Buthainah Mohammed Taha,Jumaah Dakel
511 Hussein. Biofilm Formation of KPC-producing and non
512 KPC-Producing Klebsiella pneumoniae ssp.
513 pneumoniae and Inhibitory Effect of some
514 Watery Plant Extracts on BiofilmFormation
515 Journal of University of Babylon, Pure and Applied Sciences. 2018;26(6).
516 47. Jabra-Rizk M, Meiller T, James C, Shirtliff M. Effect of farnesol on Staphylococcus aureus
517 biofilm formation and antimicrobial susceptibility. Antimicrobial agents and chemotherapy.
518 2006;50(4):1463-9.

48. Gawad WE, Helmy OM, Tawakkol WM, Hashem AM. Effect of EDTA on biofilm formation and antibiotic susceptibility of multidrug resistant uropathogenic Escherichia coli clinical isolates in Egypt. 2017.

49. Chaudhary M, Kumar S, Payasi A. Role of CSE1034 in Escherichia coli biofilm destruction. J Microb Biochem Technol. 2013;5:54-8.

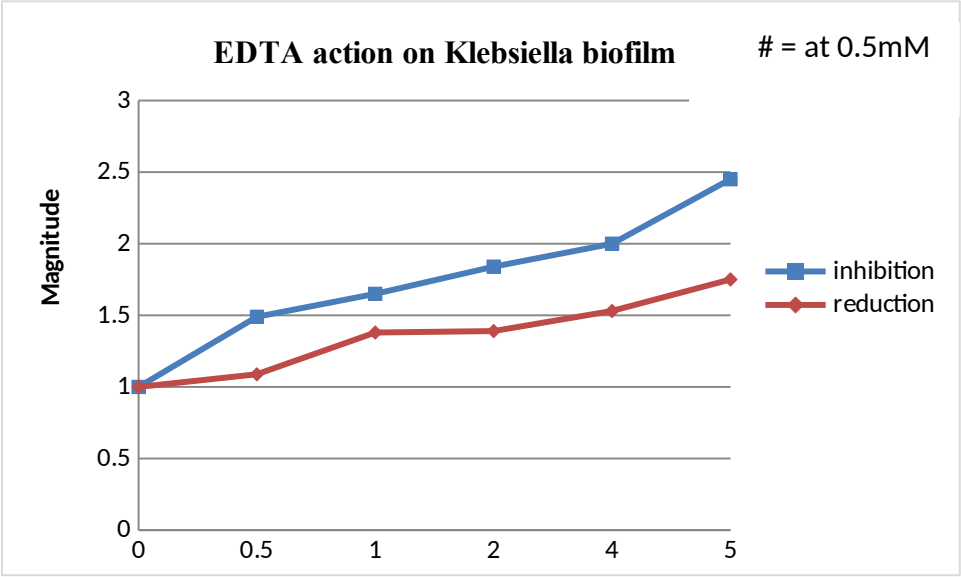
50. Guo Q, Wu Q, Bai D, Liu Y, Chen L, Jin S, et al. Potential use of dimethyl sulfoxide in treatment of infections caused by Pseudomonas aeruginosa. Antimicrobial agents and chemotherapy. 2016:AAC. 01357-16.

Figures:



Note: # = p-value <0.05

Figure 1(a)



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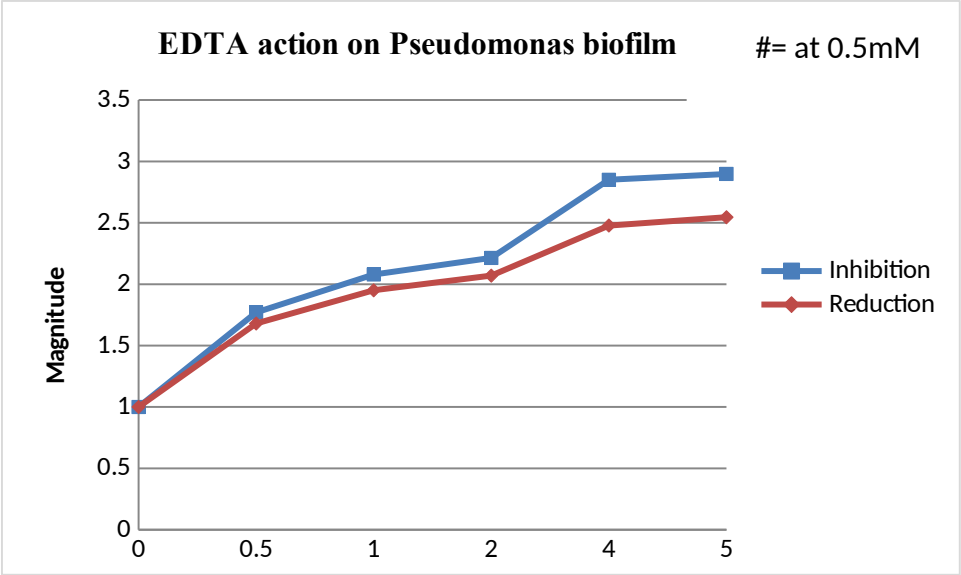

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549 Note: # = p-value <0.05

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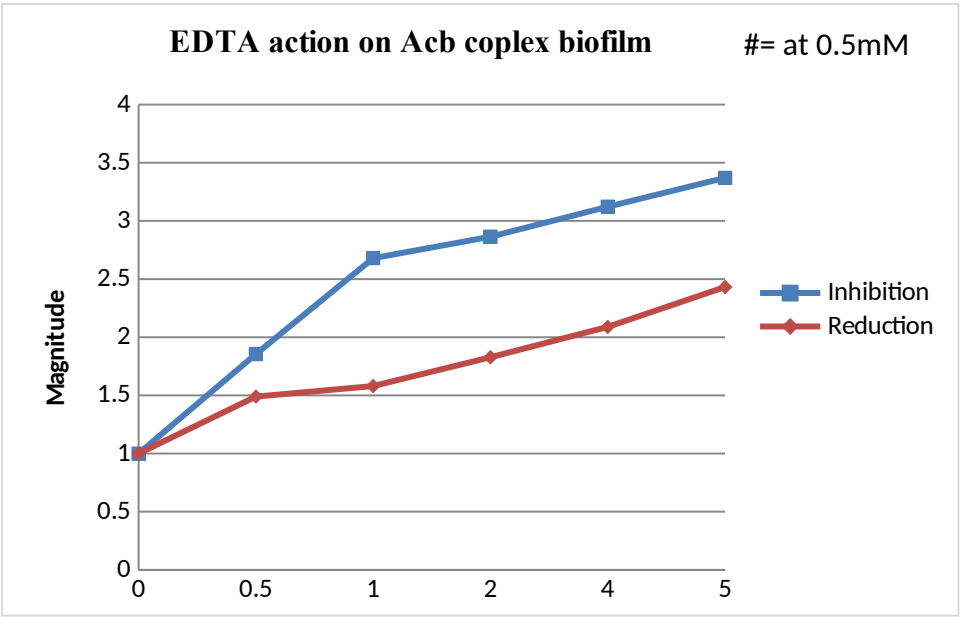

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553 Note: # = p-value <0.05
554 *Abbrebiation: Acb=Acinetobacter calcoaceticus baumannii*
555
556 **Figure 1(d)**

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558 Figure1: Biofilm inhibition and reduction by different concentration of EDTA (mM)

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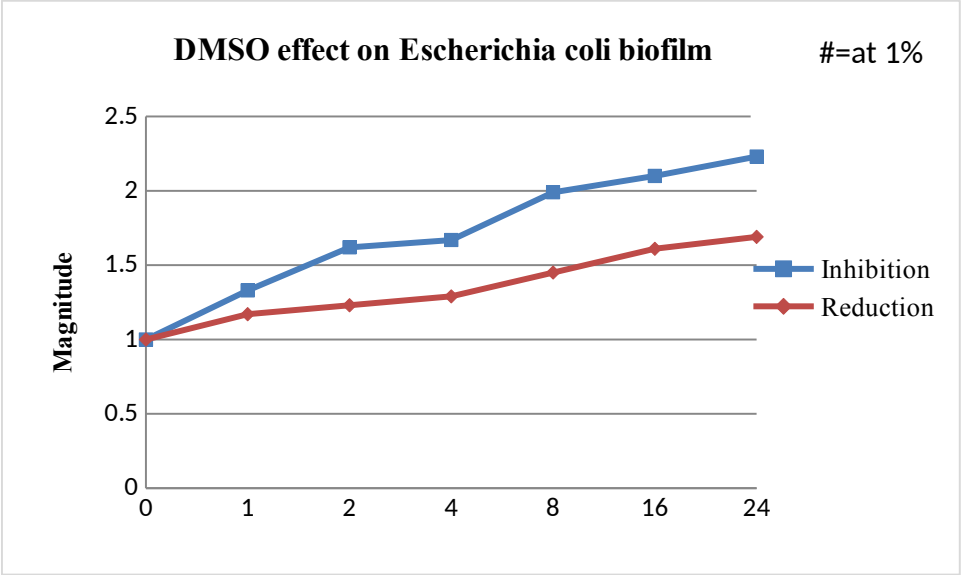

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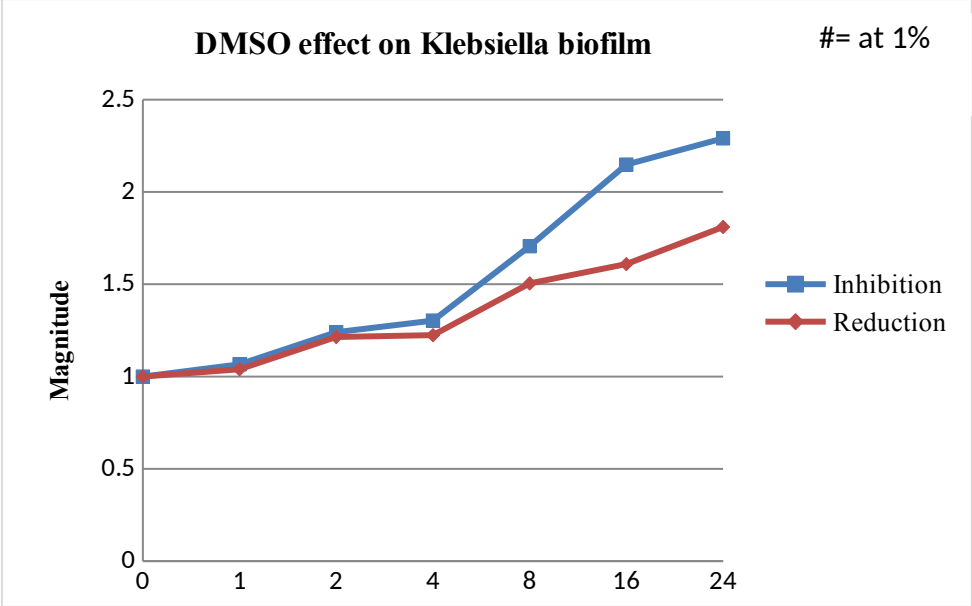

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566 Note: # = p-value <0.05

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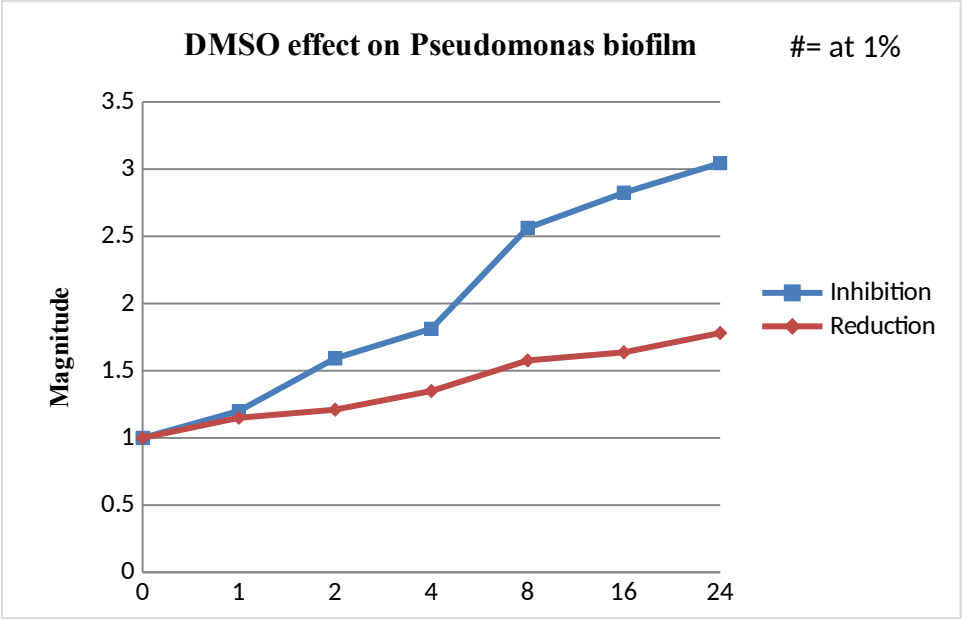

Note: # = p-value <0.05

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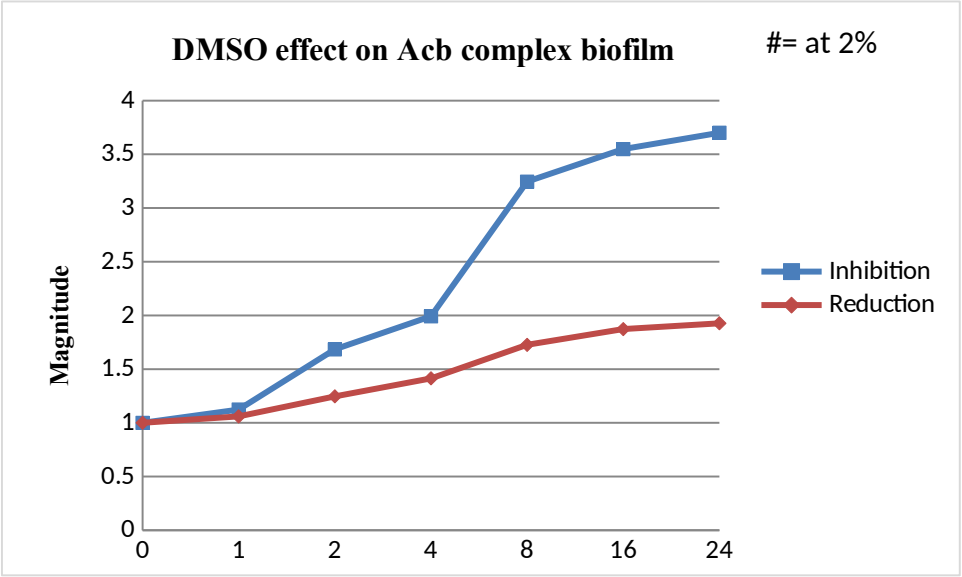

Note: # = p-value <0.05

Abbrebiation: Acb=Acinetobacter calcoaceticus baumannii

Figure 2(d)

Figure2: Biofilm inhibition and reduction by different concentration of DMSO(%)

Tables:

Table-1 Distribution of organisms in the various clinical sample

Bacterial isolates	**Urine** (N)	**Sputum** (N)	**Pus/ Wound swab** (N)	**Blood** (N)	**Body fluids** (N)	**Total** (N)
Escherichia coli	45	2	4	–	–	51
***Klebsiella* species**	11	11	9	1	–	32
P. aeruginosa	5	9	2	–	2	18
***Acb* complex**	1	7	–	1	–	9
Total	62	29	15	2	2	110

Abbreviation:*P. aeruginosa, Pseudomonas aeruginosa; Acb, Acinetobacter calcoaceticus baumannii*
Note: N denotes number of isolates

Table2: Antimicrobial resistance pattern (%) of Enterobacteriaceae

Antibiotics	***Escherichia coli*** (%)	***Klebsiella* spp.**(%)
Amoxycillin	**92.2**	-
Cefexime	47.1	**84.4**
Cefotaxime	47.1	**84.4**
Ceftazidime	43.1	84.4
Chloramphenicol	11.8	40.6
Ciprofloxacin	45.1	71.9
Levofloxacin	35.3	59.4
Gentamycin	13.7	59.4
Tetracycline	39.2	46.9
Cotrimoxazole	64.7	71.9
Imipenem	19.6	53.1
Meropenem	17.6	53.1
Tigecycline	3.9	46.9
Pieracillin-Tazobactam	11.8	59.4
Polymyxin B	0	0
Colistin Sulphate	0	0

585

586 Table3: Antimicrobial resistance pattern (%) of Non-fermenter

Antibiotics	***P.aeruginosa***(%)	***Acb* complex**(%)
Ceftazidime	44.4	**100**
Chloramphenicol	-	88.9
Ciprofloxacin	38.9	**100**

Levofloxacin	33.3	88.9
Gentamycin	33.3	**100**
Cotrimoxazole	-	88.9
Imipenem	22.2	**100**
Meropenem	22.2	**100**
Tigecycline	-	66.7
Piperacillin	**61.1**	**100**
Pieracillin-tazobactam	38.9	**100**
PolymyxinB	0	0
Colistin sulphate	0	0

587 Abbreviation: *P. aeruginosa, Pseudomonas aeruginosa; Acb, Acinetobacter calcoaceticus baumannii*
588

589 Table4: Incidence of MDR and Beta-lactamases production

Bacterial Isolates	**Total N**	**MDR N(%)**	**ESBL N(%)**	**MBL N(%)**	**KPC N(%)**
Escherichia coli	51	29 (56	18 (35.3	0 (0.0	2 (3.9

		.9 %)	%)	%)	%)
***Klebsiella* species**	32	24 (75 %)	15 (46.9 %)	8 (25%)	4 (12. 5%)
P. aeruginosa	18	6 (33 .3 %)	0 (0.0%)	2 (11.1 %)	0 (0.0 %)
***Acb* complex**	9	9 (10 0%)	0 (0.0%)	6 (66.7 %)	3 (33. 3%)
Total	110	68 (61 .8 %)	33 (30.0 %)	16 (14.5 %)	9 (8.1 %)

590 Abbreviation: ESBL, Extended Spectrum Beta Lactamases; MBL, Metallo Beta Lactamases; KPC, *Klebsiella*

591 *pneumoniae* carbapenemase; *P. aeruginosa, Pseudomonas aeruginosa; Acb*, *Acinetobacter calcoaceticus baumannii*

592 Note: N denotes for number of isolates

593

594 Table5: Organisms wise distribution of biofilm formation

Bacterial Isolates	**Total (N)**	**Stro**	**Moderat**	**Weak**	**Total (N/%)**

		ng (N)	e (N)	(N)	
Escherichia coli	51	0	0	7	7 (13.7 %)
***Klebsiella* species**	32	1	3	5	9 (28.1 %)
Pseudomonas aeruginosa	18	2	3	11	16 (88.9 %)
***Acb* complex**	9	2	1	4	7 (77.8 %)
Total	110	5	7	27	39 (35.4 %)

595 Abbreviation:*Acb*,*Acinetobacter calcoaceticus baumannii*
596 Note: N denotes for number of isolates

597 Table6: Comparison of antibiotics resistance pattern among biofilm producer and biofilm non-
598 producer

Antibiotics	**Biofilm producer**		**Biofilm non-producer**	
	No.	**(%)**	**No.**	**(%)**
Amoxycillin	6	85.7	41	93.2
Cefixime	13	81.3	38	56.7
Cefotaxime	13	81.3	38	56.7
Ceftazidime	26	66.7	40	56.3
Chloramphenicol	10	43.5	17	24.6

Ciprofloxacin	23	59.0	39	54.9
Levofloxacin	20	51.3	31	43.7
Gentamycin	20	51.3	21	29.6
Tetracycline	8	50.0	27	40.3
Cotrimoxazole	18	78.3	46	66.7
Piperacillin	10	62.5	1	50.0
Piperacillin/Tazobactam	19	48.7	22	31.0
Imipenem	20	51.3	20	28.2
Meropenem	20	51.3	20	28.2
Tigecycline	9	39.1	14	20.3
Polymyxin B	0	0	0	0

599

600 Table7: Comparison of MDR pattern and β-lactamases among biofilm producer and non
601 producer

Resistance pattern	**Biofilm producer (N=39)**	**Biofilm non-producer (N=71)**
MDR	25 (64.1%)	43 (60.6%)
ESBL	8 (20.5%)	27 (38.0%)
MBL	10 (25.6%)	7 (9.9%)
KPC	6 (15.4%)	4 (5.6%)

602 Abbreviation: ESBL, Extended Spectrum Beta Lactamases; MBL, Metallo Beta Lactamases
603 KPC, *Klebsiella pneumoniae* carbapenemase
604 Note: N denotes for number of isolates
605

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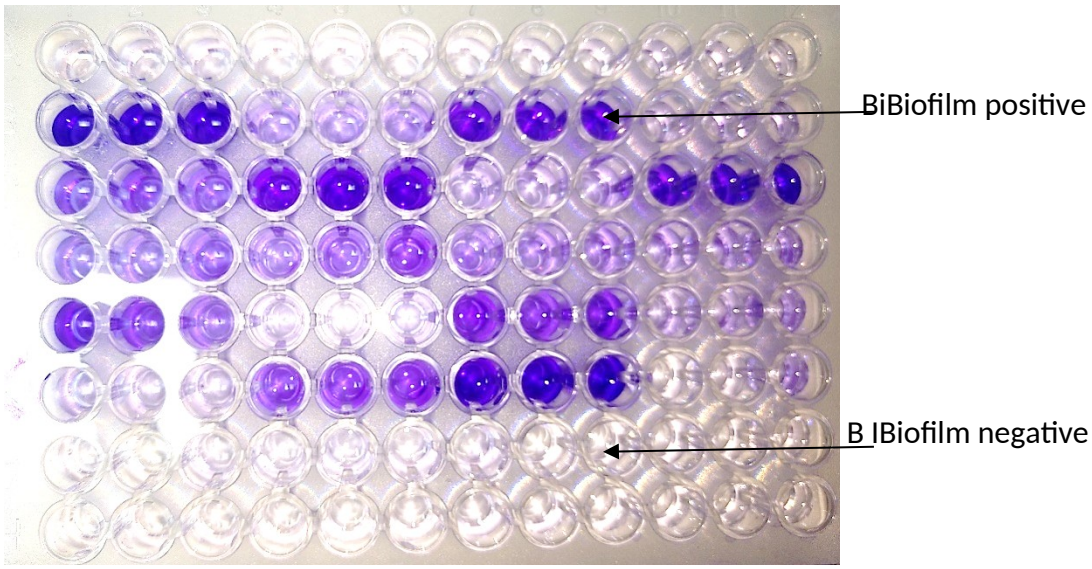
608 **Photographs:**



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610 Photograph 1: Biofilm detection by TCP method

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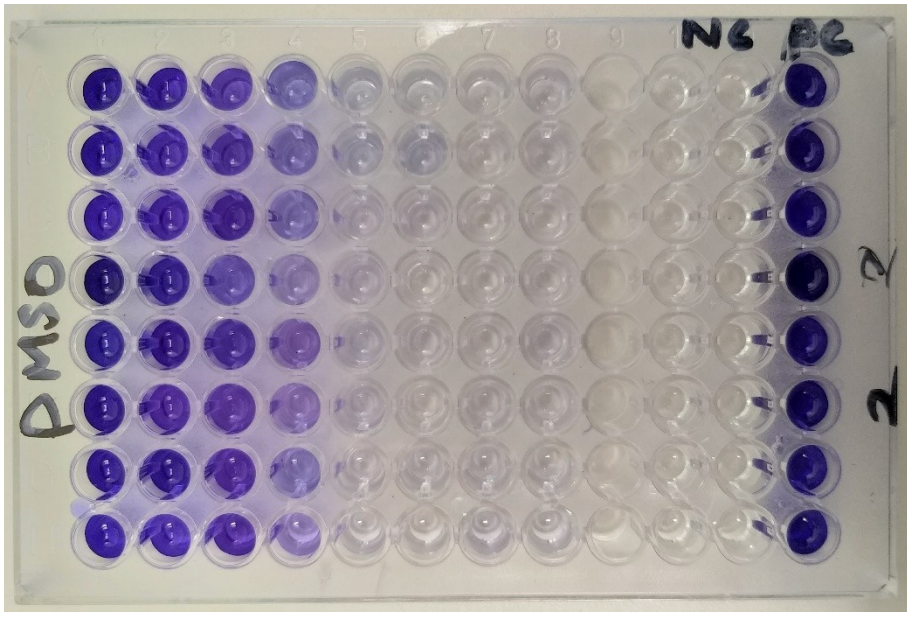

612

613 Photograph 2: Biofilm inhibition by DMSO

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