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In vitro Study on the Combined Effects of Natural Ingredients and Antimicrobial Drugs as Novel Anti Biofilm Approach

Kehkashan[1], Aqeela Ashraf[1*], Afeefa Chaudhry[1] and Roheela Yasmeen[1]

[1]Department of Biology, Lahore Garrison University, Lahore, Pakistan

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***Corresponding Author:**
Aqeela Ashraf
Department of Biology, Lahore Garrison University, Lahore, Pakistan
draqeela@lgu.edu.pk

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ABSTRACT

Biofilm forming bacteria stick to one another or to the different surfaces or interface. Biofilm formation is not a good thing in many ways as they go with low metabolic rate and passed with less number of cell divisions. **Objective:** To find some novel anti-biofilm approaches against biofilms. **Methods:** Soil and water samples were collected from four sites. Soil samples were collected from agricultural land and road side of Hudiara village, Lahore, Pakistan. However, water samples were collected from BRB canal which is situated in village Barki and from tube well of village Hudiara located in Lahore district Punjab, Pakistan. For biochemical identification of isolates different types of biochemical tests such as MR, VP, SIM (motility), H_2S, catalase, Indole and nitrate reduction were performed. **Results:** Some antibiotics and their combinations with different other antibiotics were checked and it was noticed the overall effects of antibiotics on bacterial biofilms have positive effects except disprin and Levofloxacin. While, Ciprofloxacin was found as an effective antibiotic. Combination of ciprofloxacin and disprin was used in order to remove the biofilm and it worked well to remove the biofilm. **Conclusions:** Different antimicrobial medications, all-natural compounds, and combinations of various antibiotics, including ciprofloxacin-disprin, clarithromycin-moxifloxacin, and certain all-natural ingredients like honey, ginger, and lemon juice, were utilized to remove bacterial biofilms. Thus, it can be said that most of the combinations produced better biofilm removal outcomes than the individual elements did.

INTRODUCTION

Biofilms are defined as a spreadsheet-enclosed population of bacteria combined with one another and to outsides or with the boundaries. Biofilms contain the aggregates of microorganisms [1]. Biofilms present in the natural environments are usually stick to each other or to the boundaries of surfaces. Since the first description about bacterial biofilms, their real importance has gradually emerged [2]. During the fifteen decades, ensuring the discoveries of Louis Pasteur it has come to be interestingly clear that the biofilms possessed a noticeably different growth phase of bacteria that is extremely diverse from the planktonic growth phase being studied thoroughly [1]. Antibiofilm approaches are techniques to remove biofilms. Different methods can be used to remove biofilms as some of the bacterial biofilms have serious problems and in fact proved harmful in different aspects of life. Some bacterial colonies are harmful in clinical aspect and some are not beneficial in food industry. Some biofilms are resistant to different antibiotics so there is a need to find a way to remove these harmful biofilms [3]. Different anti-biofilm techniques have been used for biofilm removal e.g. nanotechnology, some enzymatic effects like aptamers and some antibiotics have been used previously to remove biofilms but many novel techniques are needed in order to remove the biofilm or to stop the biofilm formation [4]. Biofilms influence the human beings in several methods for instance these can be produced in medical, natural, and industrial locations. For example, development of biofilms on the medical instruments, like implants or catheters regularly is tough to cure the chronic syndromes [5, 6]. Furthermore, some diseases have been linked with the biofilm production on human surfaces like skin, urinary

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tract and teeth. Though, biofilms on the external surfaces of the human beings are not continuously harmful. For example, dental plaque include lots of kinds as well as colony production is a normal thing and not as harmful as the other types of biofilms for human beings [7]. Prevalence of the biofilms is important problem in the food industry and in medical. Main foodborne disease causing microbes like *E.coli, Salmonella spp.*, *Listeria monocytogenes* and *Campylobacter jejuni* can produce biofilm and can be a major challenge in food safety in food industries [8]. Resistance of microorganisms has attained permanent or temporarily capability of an organism while multiplying in situations and would eradicate or obstruct the other members of same strain. Antibiotics resistance is well acknowledged; though, disinfectants, food preservatives, and resistance toward disinfectants are relatively under explored. In order to control gene expression of the biofilm forming bacteria or other microorganisms in coordinated manners as well as facilitate colonies of bacteria is one of trademark methods on behalf of treatments in bacterial films as well [9]. Biofilm in which cells of microorganisms attach to each other and to the surface area of others has numerous environmental and economic benefits containing oil recovery, medical implants, paper making, drinking-water distribution, food processing and metal working [10]. Antimicrobial drugs have a variety of useful genetic materials, enzymes and some additional cellular loci. Though, because of inherited interactions and intrinsic divergences like special cell cover configuration as well as non-susceptible protein, various bacteria respond contrarily to the bactericides. The bacterial biofilm has increased the resistance of antibiotics as well as involved in several insistent disorders. In biofilm, there are various methods for resistance towards the antibiotics as well [11]. Major objective of this study was to make biofilm and find some novel anti biofilm approaches. Antibiofilm approaches which were used in this study were use of antibacterial drugs, natural ingredients and the combined effects of antibiotics and natural ingredients. Natural ingredients that was used in this study were honey, ginger extract and water. Antibiotics that used were ciprofloxacin, moxifloxacin, clarithromycin, levofloxacin and disprin.

METHODS

Soil and water samples were collected from four sites. Soil samples were collected from agricultural land and road side of Hudiara village, Lahore, Pakistan. However, water samples were collected from BRB canal which is situated in village Barki and from tube well of village Hudiara located in Lahore district Punjab, Pakistan. Soil samples was sieved with 2mm of sieve and physiochemical characteristics such as pH and temperature were also noticed after mixing of 0.2 g of soil in 2ml of distilled water. Samples were serially diluted and hundred microliters of each sample was taken and spreaded onto nutrient agar plates through spreader. Inoculated plates were incubated for 48 hours at 37°C. After incubation colonies were observed. After 48 hours of incubation viable colonies were picked and were further purified by streak plate method. Macroscopic characteristics of selected bacterial isolates were observed according to their colony shape i.e. circular to filamentous, size i.e. from pinpoint to moderate, pigmentation, elevation, texture and gram's nature. Moreover, Gram staining was performed to separate gram negative and gram positive bacteria. For biochemical identification of isolates different types of biochemical tests such as MR, VP, SIM (motility), H2S, catalase, Indole and nitrate reduction were performed. Nutrient broth was prepared according to standard microbiological method for the formation of biofilm. Selected bacterial isolates were than inoculated in test tubes containing nutrient broth and was incubated for 4-6 days at 37°C temperature in an incubator. After 6 day of incubation broth was discarded. Then crystal violet was added in test tubes for 20 minutes at room temperature. Test tubes were washed slowly with sterile water. Purple colored ring formation around the test tubes indicates biofilm formation. Formation of biofilm was further confirmed by using centrifugation method. Broth containing bacterial isolates was poured into the Eppendorf's and centrifuge machine was set up to 1000 rpm for 15 minutes and temperature was set up to 37°C. After 15 minutes the bacterial colonies were clearly noticed at the bottom of the Eppendorf's [12]. Bacterial cells were collected for further processing and supernatant was removed. The pellets at the bottom ensured the formation of biofilm. Different natural ingredients, antibacterial drugs and different combination of the drugs were used to remove bacterial biofilms. In natural ingredient honey, lemon juice and ginger extract were used. For honey one gram of honey was mixed with 10 ml of distilled water and used against biofilm containing test tubes. After few minutes' results were recorded. In case of lemon one ml of fresh lemon juice was mixed with 9 ml of distilled water and was added in a test tube containing biofilm and results were observed. Similarly, extract of ginger was used to remove biofilm and after few minutes' results were recorded. Half gram disprin and ciprofloxacin powder were mixed in 10 ml of distilled water. The solution was shaken well and mixed properly. The solutions were added in test tubes containing biofilm and results were recorded properly. Combination of disprin and ciprofloxacin were made to check the effect of biofilm. 5 ml of disprin solution and 5 ml of ciprofloxacin solution were mixed together and a combination of these two drugs were

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added in test tubes containing biofilm and results were recorded. Similarly, moxifloxacin drug and combination of moxifloxacin and levofloxacin were made and used against biofilm forming bacteria and results were recorded. One gram of clarithromycin powder was mixed in 10 ml of distilled water. The solution was shaken and mixed well. This solution of antibacterial drug was used as an antibiofilm agent and was added in test tubes containing biofilm.

RESULTS

Microscopic characteristics were observed under microscope. AS2 and BR2 appeared to be cocci species whereas AS3, RS3, BR3, TW1 observed to be bacillus species as shown in table 1.

Isolate ID	Microscopic Characters
DAS2	Cocci
As3	Bacilli
RS3B	Bacilli
R2B	Cocci
R3T	Bacilli
W1	Bacilli

Table 1: Microscopic characterization of selected bacterial isolates

For gram positive isolates positive results were shown for Catalase Vogues Proskauer (formation brown ring on top) and nitrate reduction test (color change to orange) and negative results were noticed for Indole tests (no cherry red ring formation and SIM (no motility) tests. Red color in tests tubes indicated positive result for Methyl Red (MR) tests and these tests indicated bacterial specie which were cocci. While for gram negative isolates; positive results were shown for Catalase by the formation of bubbles, SIM (sulphide indole motility) tests, nitrate reduction and Vogues Proskauer (VP) showed positive results whereas negative results were shown for Methyl Red (MR), Indole and H2 S indicated as bacilli specie and was observed in table 2.

Isolates ID	MR	VP	Indole	Catalase	Nitrate reduction	SIM	H2S	Specie
AS2	+	+	-	+	+	-	-	Cocci
AS3	-	+	-	+	+	+	-	Bacilli
RS3	-	+	-	+	+	+	-	Bacilli
BR2	+	+	-	+	+	-	-	Cocci
BR3	-	+	-	+	+	+	-	Bacilli
TW1	-	+	-	+	+	+	-	Bacilli

Table 2: Biochemical tests of bacterial isolates

Biofilm was formed in N-broth. To ensure the biofilm formation N-broth was discarded and crystal violet staining was done. After staining of crystal violet ring formation on the test tube ensure the formation of biofilm. All bacterial isolates formed biofilm production and positive results were observed as shown in table 3.

Sr. no.	Bacterial isolates	Biofilm formation
1	AS1	-ve
2	AS2	+ve
3	AS3	+ ve
4	AS4	- ve
5	AS5	- ve
6	RS1	- ve
7	RS2	- ve
8	RS3	+ ve
9	RS4	- ve
10	RS5	- ve
11	BR1	- ve
12	BR2	+ ve
13	BR3	+ ve
14	BR4	- ve
15	BR5	- ve
16	TW1	+ ve
17	TW2	- ve
18	TW3	- ve
19	TW4	- ve
20	TW5	- ve

Table 3: Biofilm formation with selected bacterial isolates

Honey, lemon and ginger extract were used to remove biofilm. Table 4 shows the overall result of removal of biofilms through natural ingredients. Pure lemon extract was used for the removal of biofilms but it did not prove helpful in order to remove biofilm. While solution of lemon extract and water was also used in order to remove bacterial biofilm, it proved helpful for the removal of biofilm. However, honey and ginger extract were found useful as an anti-biofilm agent and it showed positive results. Some antibiotics and their combinations with different other antibiotics were checked and it was noticed the overall effects of antibiotics on bacterial biofilms have positive effects except disprin and Levofloxacin. While, Ciprofloxacin was found as an effective antibiotic. When the biofilm was treated with the ciprofloxacin the biofilm was removed immediately. Then a combination of ciprofloxacin and disprin was used in order to remove the biofilm and it worked well to remove the biofilm. Clarithromycin showed positive results as an anti-biofilm agent. Similarly, Moxifloxacin used to remove the biofilm and proved an effective drug against removal of biofilms. Levofloxacin showed positive results when used in combination.

Antibiofilm agent	Result
Honey	Negative
Ginger extract	Positive
Lemon extract	Negative
Lemon extract+ Water	Positive
Antibiofilm agent	Result
Disprin	Negative
Ciprofloxacin	Positive

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Moxifloxacin	Slightly Positive
Levofloxacin	Negative
Clarithromycin	Positive
Combined Effects	
Disprin + Ciprofloxacin	Positive
Levofloxacin + Moxifloxacin	Positive
Clarithromycin + Levofloxacin	Positive
Honey+ Ciprofloxacin	Positive
Lemon + Clarithromycin	Positive
Ginger + Moxifloxacin	Positive

Table 4: Natural products and antibiotics used for the removal of biofilm

DISCUSSION

Main objective of this study was to find some novel anti-biofilm strategies and for this purpose first step of this study was to produce some bacterial biofilms. In this study different natural ingredients and some antibiotics along with some combinations of these antibiotics were used in order to remove the biofilms. Natural ingredients which were used in this study were honey, lemon and ginger. The drugs which were used in this study to remove biofilms were disprin, ciprofloxacin, moxifloxacin, levofloxacin, clarithromycin and their combinations with one another. For the removal of biofilms different antibiotics and natural ingredients were used. First of all most commonly used drug (disprin) was used to check its efficiency against the biofilms but it did not prove helpful and it seemed that biofilm is resistant to disprin because disprin is not an antibacterial drug but an NSAID (Non-steroidal anti-inflammatory drug) so it will not remove the bacterial biofilm [13]. Ciprofloxacin proved effective against biofilms independently and in combined form as well which means biofilm has no resistance against the ciprofloxacin. Ciprofloxacin not only removed biofilm independently but also make disprin effective against biofilms when it was combined with it and also reported by Karaca et al., [14]. Same is the case with clarithromycin which independently did not prove effective against the removal of biofilms but when used in combination with levofloxacin then it showed positive results as reported by Marchese et al., [15]. Most of the antibiotics showed positive results and prove useful as anti-biofilm approaches. This is because of the reason that biofilms are actually the colonies of bacteria and antibiotics are effective against bacteria [16]. So when these antibiotics were used against the bacterial biofilms then some of them immediately removed biofilms and some took time to perform their activity [17]. Hence the activity of different anti-biofilm agents was checked according to rate of time as well [18]. Natural ingredients were also used to check their efficacy against the biofilms. First natural ingredient which was used was honey as it works well against biofilms [19] but when we treat it in concentrated form against biofilms it did not remove them which mean it did not have anti-biofilm ability in it. Honey can be effective against biofilms in its diluted form. To increase the effectiveness of honey against biofilms it was combined with antibiotic and when it was combined with ciprofloxacin it showed very good anti-biofilm activity as reported by Rendueles et al., [20]. Lemon extract was used to remove biofilms which did not removed it in its concentrated form but when the lemon extract was diluted and mixed with water then at once its efficiency increased as well [21]. Then concentrated and diluted both solutions of lemon extracts were mixed or combined with the moxifloxacin. Diluted solution of lemon extract removed the biofilm while the concentrated solution did not show any response here again which means antibiotic has no effect on lemon extract to make it effective as anti-biofilm agent [22]. Ginger extract was then used to remove biofilms. Ginger extract has natural ability to remove any kind of bacteria or bacterial colonies either these are in the form of biofilms or not [23]. And it was seen practically when ginger extract was used against the biofilms it immediately removed it and in combination with antibacterial drugs its efficacy increased and where it was taking some time to remove biofilm independently there when combined with antibiotic its efficacy increased and it removed biofilm within few minutes [24]. Mostly combined effects of natural ingredients and antibacterial drugs showed positive results as compared to the results in independent form. But it was just one way to remove biofilms, there is need to find more ways or anti-biofilm approaches to get rid of these as these are very dangerous and harmful in different aspects for many living things and specially for human beings, so more work and more researches are required on this topic or some different topics related to this study [25].

CONCLUSIONS

For the removal of bacterial biofilms different antimicrobial drugs, natural ingredients and combinations of different antibiotics like ciprofloxacin- disprin, clarithromycin-moxifloxacin and some natural ingredients like honey, ginger and lemon juice were used. It can thus be concluded that most of the combinations showed positive results for biofilm removal as compared to the ingredients used independently.

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