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EFFICACY OF MEDICINAL PLANTS AGAINST BIOFILM FORMING BACTERIA PLAYING IMPORTANT ROLE IN MULTIDRUG RESISTANCE

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ABSTRACT

Background: Biofilms are the bacterial aggregates adhered to the solid surfaces and are self-initiated, protective exopolysaccharide matrix associated with the severity of diseases. These may also increase the risk of Multi Drug Resistance among the bacterial strains by surrounding the bacteria which protects them from various environmental stresses, antimicrobials, disinfectants, and the host immune system. Due to these characteristics, it seems extremely difficult to eradicate the bacteria from the host.

Objective: The study is focused on the destruction of these biofilms formed by the pathogens (*E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus* and *S. typhi*) contributing to the resistance and persistence of bacteria in the host.

Methods: The *in vitro* biofilm formation assays: Adhesion assay, Tube-method and Microtitre plate assay were performed for 5 previously characterized clinical MDR isolates and their respective standards (MTCC). Also, these strains were further treated with antimicrobials, plant extracts and their combinations to study their effect in the biofilm destruction.

Results: All the clinical as well as standard strains were found biofilm forming by tube and microtitre plate assay, The clinical isolates were strong biofilm former than standard isolates. A

total of 7 medicinal plants were studied for the destruction of bacterial biofilms. The highest destruction of biofilm was observed with *Rouwolfia serpentine* extracts followed by *Eclipta prostrata* extracts and the least effective plant extract was of *Messua ferra*. The highest destructive effect was observed on the pathogens of *Enterobacteriaceae* family including *S. typhi*.

Conclusion: It has been concluded from the study that medicinal plants have bioactive compounds that can destruct the biofilm formation (bacterial aggregation) and also help in the exclusion of bacterial strains that cause persistence infections in humans. Therefore, the bioactive compounds may play important role in the treatment of persistence infection and treating MDR cases.

Keywords: Biofilm formation; Multi drug resistance; bioactive compound; Medicinal plants; Biofilm destruction.

INTRODUCTION

Biofilm are self-organized grouping of micro-organism which produces a matrix of exopolysaccharide; the structure of biofilm is a great complex of polymers which can change with the variations in nutritional gradient as well as change in the environmental conditions. Exopolysaccharide layers act as a protective layer which protects the microbial community from antimicrobial agents. Exopolysaccharide mainly constitute polysaccharide and other compound such as protein lipid, DNA humus substance [1]. The exopolysaccharide (EPSs) function as construction material for the biofilm formation and constitute 50% to 90% of a biofilms total organic matter [2]. Biofilms are complex aggregates of bacterial cells attached to the surface and enclosed in a

polymer matrix [3]. Biofilm act as city of microbes and the EPS as the house within the biofilm [4]. Bacteria inside the biofilm exist in dormant phase and exhibit different phenotypes distinct from the normal bacteria due to this reason bacteria exhibit a resistance against different type of environmental stress [5]. Biofilms are often responsible for chronic illness and hospital-acquired (nosocomial) infections. Bacterial biofilm formation and aggregation consist of reversible and irreversible stages based on different factors. The bacterial aggregation is driven by gravitational forces, temperature, pH, nutrients, and Brownian movement but the motile bacteria with the help of their flagella overcome these forces [2]. This explains the great interest in studying

biofilms and the search for new naturally or chemically synthesized compounds capable of blocking its formation. The aim of this work was to study the role of medicinal plants against the human pathogens (*S. aureus*, *S. typhi*, *K. pneumoniae*, *P. aeruginosa*, *E. coli*) which forms biofilm and is responsible for severe diseases. The main objective of the study was to destruct the biofilm formation with the help of medicinal plants.

MATERIALS AND METHODS

Collection of samples

The laboratory adopted isolates of 5 pathogenic MDR bacteria were obtained from Molecular and Immuno-Parasitology Research Laboratory to study the biofilm formation among these bacteria and the standard strains of *E. coli* (MTCC-739), *K. pneumoniae* (MTCC-39), *S. aureus* (MTCC-737), *P. aeruginosa* (MTCC-741), and *S. typhi* (MTCC-734) were collected from IMTECH, Chandigarh. The microorganisms were subcultured in nutrient broth and incubated at 37°C for 24 hrs before the experiment. A total of 7 authenticated medicinal plants, i.e., *Messua ferra*, *Vitex negundo*, *Andrographis paniculata*, *Eclipta prostrata*, *Rouwolfia serpentina* and *Gloriosa*

superba were collected from different areas of Himachal Pradesh.

Enumeration of Viable Cell Count

The overnight broth culture was serially diluted with normal saline up to 10⁻⁶ dilution and 100µl of each dilution was spread on to Nutrient agar (HI Media) plates and incubated overnight at 37°C. After 12-18 hours incubation, the number of viable colonies was counted using total viable plate count method (Prescott, 2013).

Detection of biofilm formation

The detection of biofilm formation in the pathogenic bacteria was performed by using the two basic techniques, Tube method and the Microtitre plate assay (Adherent assay). The Tube method [6] was used for the qualitative detection of biofilm formation. A loopful of organisms was inoculated in 10 mL of trypticase soy broth with 1% glucose in test tubes. The tubes were incubated at 37°C for 24 hours. After incubation, tubes were washed with phosphate buffer saline solution (pH 7.2), dried and stained with crystal violet (0.1%). Excess stain was washed with deionized water and tubes were dried. The Biofilm formation was considered positive after observing a visible film lined the wall and the bottom of the tube. The biofilm formation was categorized in three

forms 1-weak/none, 2-moderate and 3-high/strong. The experiment was performed in triplicate and repeated three times. The microtitre adherent assay was performed for the quantitative analysis of biofilm formation. Similarly, an overnight culture of pathogenic strains and their respective standards on TSA (Tryptic Soy Agar) was diluted 200-fold with culture medium (TSB+1% glucose), of which 200µl was added to the wells of a 96-well polystyrene microtitre plate and Incubated at 37°C for 24h. After the incubation period wells were aspirated carefully and washed 3-4 times with PBS (pH 7.2). Cells were emptied, fixed and quantified for biomass production. The cell density was determined at 490nm with a microtitre plate reader (BioTek ELx800). Biofilm formation in each well was analyzed by 0.1 safranin/crystal violet staining methods [7]. Each experiment was performed at least three times and in each experiment the samples were prepared in six wells. Experiment was performed in triplicate and repeated three times, the data was then averaged and standard deviation was calculated. To compensate for background absorbance, OD readings from sterile medium, fixative and dye were averaged and subtracted from all test values. The mean OD

value obtained from media control well was deducted from all the test OD values.

Effect of medicinal plant extracts on biofilm Inhibition:

The biofilm was formed and after the confirmation of biofilm formation, the similar solution was pipetted out in the 96 well plates. Before the staining process the effective concentrations of plant extracts (5mg/ml), antibiotics (0.500mg/ml) and negative control was added as per respective wells. The plates were incubated at 37°C for 24hrs. Disruption of biofilm was analyzed by staining with crystal violet / safranin (1%).

The % inhibition was calculated by using the following formula:

$$\% \text{ inhibition} = \frac{(\text{OD of control} - \text{OD of media control}) - (\text{OD of Test} - \text{OD of extract control})}{\text{OD of negative control} - \text{OD of media control}} \times 100$$

RESULTS

A total of 10 bacterial strains (both clinical as well as standard) were analyzed for the biofilm formation and the destruction of these biofilms using methanolic extracts of 7 important medicinal plants. The result showed the standard strain of *S. typhi*, *K. pneumoniae* and *S. aureus* were not able to form biofilms on the abiotic surface while, *P. aeruginosa* and *E. coli* were found moderate biofilm producers. However, the clinical

strains of *S. typhi*, *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *S. aureus* were strong biofilm producers (Table1, Figure 1).

The maximum production of biofilm was observed in clinical isolates of *K. pneumoniae* followed by *S. typhi* and *P. aeruginosa* while the lowest biofilm formation was observed in standard strain of *S. typhi* and *K. pneumoniae*. The results clearly showed that the MDR strains were stronger biofilm producers than those of the standards obtained.

It was observed from the Figure 2 that the methanolic extracts of *R. serpentina* showed maximum destruction of biofilm in all the pathogens followed by *P. roxburghii* and *E. prostrata*, while the lowest effect was observed from the extracts of *M. ferra*. It was also found that the extracts of *R. serpentina* and *P. roxburghii* showed

highest destruction on the biofilms formed by the MDR strains of enteric bacteria (*S. typhi* and *E. coli*). The study also found that biofilms formed by the gram negative bacteria were destructed significantly in higher manners as compared to the gram positive bacteria. It was also analyzed that most of the methanolic extracts were found to be highly destructive against the biofilms produced by the MDR strains of *S. typhi* which is the most restrictive human pathogen (Figure 2). The results showed that the most effective antibiotics for the destruction of biofilm among the MDR isolates were found to be Erythromycin and streptomycin. While comparing to the antibiotics the most effective destructive plant extract was of *Rouvolfia serpentina* and no biofilm formation was observed in the negative control (TSB).

Figure 1: The graphical representation showed the biofilm formation among the human pathogens (bacterial strains)

Table 1: Biofilm formation among the standard and clinical strains of pathogenic bacteria

Biofilm formation in clinical and standard strains of bacteria	
None	<i>S. typhi</i> standard, <i>S. aureus</i> standard, <i>K. pneumoniae</i> standard
Moderate	<i>P. aeruginosa</i> standard, <i>E. coli</i> standard
Strong	<i>S. typhi</i> clinical, <i>P. aeruginosa</i> clinical, <i>S. aureus</i> clinical, <i>K. pneumoniae</i> clinical, <i>E. coli</i> clinical

Figure 2: Effect of methanolic extracts of various medicinal plants (*Messua ferra*, *Vitex negundo*, *Andrographis paniculata*, *Eclipta prostrata*, *Rauwolfia serpentina*, *Gloriosa superba*) on the biofilm formed by pathogenic bacteria (*E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *S. typhi*)

DISCUSSION

Some bacterial disease considered to be communicable for as long as the infected person remains the carrier for the disease. As per the availability of major treatment and prevention efforts, millions of new bacterial cases evolve worldwide every year. The bacterium (*E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *S. typhi*) colonizes systematically and remains there for long after symptoms subside to be a reservoir for the further spread of the disease [8]. Bacteria that grow as biofilm encased in a self-

produced matrix are protected from host defenses and often exhibit reduced antibiotic susceptibility, contributing to the persistence of biofilm infections [9].

Biofilm formation played a very significant role in establishing long-term colonization of bacterial cells that continuously shed for extended periods [10]. In the present study, we tried to find a correlation between MDR and the Biofilm production capability of isolates and the effect of plant extract on biofilm producing bacteria. It was reported earlier that the

strong biofilm producing bacteria shed upto a maximum period of 50 days (average 32.25 days) in post-infection cases of whereas the non-biofilm producers was not even able to shed upto 17 days (average 13.28 days). This property of theses strong biofilm producers leads to the development of antibiotic resistance that can be up to 1,000-fold greater than planktonic cells [11]. Multi drug resistance to several antibiotics has become a major public health problem worldwide as it reduces the effectiveness of treatments [12]. The bacteria in a biofilm are 1,000- fold more resistant to antibiotic than the same organism that is grown planktonically [3, 13]. This leads to the research on medicinal plants as potential sources of new and effective antimicrobials with novel modes of action. Although in some cases the active constituents may occur in low concentrations, but plant extracts may contain secondary metabolites that represent a superior source of antimicrobial compounds than synthetic drugs [14]. In the present study, a few medicinal plants (*Messua ferra*, *Vitex negundo*, *Andrographis paniculata*, *Eclipta prostrata*, *Rouvolfia serpentina* and *Gloriosa superba*) of therapeutic potential have been studied to overcome the problem of multidrug resistance in the biofilm producing

pathogens. The microplate assay was used for the turbidometric analysis of biofilm *in vitro*. Biofilm of all the bacterial strains were formed and the optical density was analyzed to detect the amount of biofilm formed.

The plant extract of *Rouvolfia serpentina* found to be most effective in the destruction of biofilm formation by the human pathogens under study. The biofilm inhibition assay tests the effect of extracts on the formation of biofilm, and not on penetration of compounds into pre-formed biofilms. This could explain why the biofilms in the present study are highly sensitive to small concentrations of the natural compounds. These studies will need to be exposed in comparison to the planktonic phase bacteria. The biofilm forming strains showed increased antimicrobial resistance to several classes of antibiotics as compared to the planktonic bacterial strains (non-biofilm producers). Once the antibiotic level drops, the persisters may multiply, explaining the relapsing nature of biofilm infections. Our findings provide valuable information in the regards of correlation between the formation of biofilm and the prevalence of MDR strains and the effect of plant extract on biofilm.

CONCLUSION

It was inferred from the present investigation that the standard strains of *S.*

typhi, *E. coli* and *K. pneumoniae* were very poor biofilm producers while, the standard strains of *P. aeruginosa* and *S. aureus* formed moderate biofilms whereas, the clinical strains of all the human pathogens taken under study were the strong biofilm formers. It has also been suggested from the study that the extracts of *R. serpentina* can be used in the drug formulations against the biofilm destruction and to be bactericidal against the pathogenic bacteria which forms protective biofilms. This experimental knowledge of the antimicrobial properties of plant species may contribute to the development of drugs against pathogenic microorganisms and suggested that when these extracts of plant origin will be combined with the synthetic drugs may act in the synergism to establish golden standards in the inhibition of protective biofilms of resistant bacteria.

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