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IN VITRO ANALYSIS OF PLANT EXTRACTS AGAINST BIOFILM FORMATION BY *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Bacteria forms biofilms by using quorum sensing. In biofilm different type of bacteria makes group with each other due to that maximum antibiotic fails to degrade it, that's why that has become the topic of research, in this my present work I have cultured *Pseudomonas aeruginosa* bacteria to form biofilm and it was tried to degrade by different natural extracts.

Key words: Metabolites, Quorum Sensing, Antimicrobial, Biofilm, *Pseudomonas aeruginosa*.

INTRODUCTION

Biofilms can be characterized as bacteria attaching their own synthesis to aggregate surfaces in a hydrated polymer matrix. The development and inherent resistance of these sessile populations to antimicrobials is the cause of many recurrent and chronic bacterial infections **[1,2].** These bacterial biofilms are abundant on most of nature's wet surfaces and can trigger environmental issues. A lot of scientists have sought to describe biofilms in their own words over the evolution of time. Van Leeuwenhoek first discovered the minute living creatures in lenses which he had created. He studied various samples such as plant leaves, saliva, faeces, soil samples and dental plaque, and named these minute species "animalcules." In 1976, Marshall found "very tiny extracellular polymer fibrils" which he described as bacterial attachment factors to various biotic and abiotic surfaces. In short, biofilms can be identified as a bacterial derived sessile population defined by cells that are irreversibly attached to a substratum and deposited in a matrix of extracellular polymeric substances that they have formed, and exhibiting an altered phenotype with terms of growth rate and gene transcription **[3,4,5].** The adhesion activates the expression of genes regulating the development of bacterial components required for adhesion and the formation of biofilms, focusing on the fact that the process of the formation of biofilms is controlled by different genes transcribed during the initial adhesion. In recent years, an increased emphasis has been put on the biofilm producing capacity of a group of clinically important bacteria, causing high mortality, abbreviated with the acronym ESKAPE that stands for *Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter spp*. **[6].**

Biofilm can be described as an accumulation of one or more microbial species which abide to, but also to, different surfaces. These bacterial species are included and interlinked within an organic structure assigned with the term extracellular polymeric substance (EPS), also known as "slime," composed of protein substances, polysaccharides, and DNA **[7].** Biofilms develop several common features on various solid areas , e.g. heterogeneous structure, multiple interrelationships between the components, diverse genetic features, and strong irreversible attachment to living or inanimate surfaces.

The increasing prevalence of pathology linked to biofilm can be explained by the growing incidence of exploratory and/or therapeutic procedures involving the use of implanted medication. The principal motives for biofilm creation are defense **[8],** colonization **[9],** and culture.

Table 1: Practical approach for biofilm detection [**10, 11]**

Techniques	**Methods**
Detection of biofilm by *in vitro* techniques	Microtitre plate assays *In vitro* flow displacement bi systems *In vitro* microfluidic devices *In vitro* tissue Culture Plate, Tu Congo Red Agar
Detection of biofilm by *in vivo* techniques	Non – vertebrate animal models Vertebrate animal models *In vivo* tissue – biofilm models *In vivo* device related infections – biofilm models
Quantification of microbial biofilm	Microtiter plates Techniques to determine the total biofilm biomass The number of viable sessile cells only The amount of extracellular polymers in the biofilm Roll-plate technique for central venous catheters
Visualization of microbial biofilm	Scanning electron microscopy Epifluorescence microscopy Confocal laser scanning microscopy
Bacterial recovery	Sonication and isolation Fluorescent in situ hybridization Metabolomic profiling and genomic analysis Multiple diagnostic techniques Polymerase chain reaction

MATERIALS AND METHODS

Microscopic analysis of *P. aeroginosa* biofilm in NB and M63 media:

The *P. aeruginosa* was inoculated in both media and additional coverslips were added to the media, which provides the surface for the biofilm formation. Further the incubation was provided for the biofilm formation and the visualization of biofilm was carried out by performing the crystal violet staining and observation carried out under light microscope at 40X resolution **[12]**.

Biofilm growth of *P.aeroginosa* by tube method:

The *P. aeruginosa* was inoculated to both media in glass tubes, which provides the surface for the biofilm formation. Further the incubation was provided for the biofilm formation and the visualization of biofilm was carried out by performing the crystal violet staining and observation carried out under light microscope at 40X resolution **[13]**.

Preparation of plant extracts for biofilm inhibition assay:

Five plant samples were collected to check their anti-biofilm activity against *P. aeroginosa*. The plant samples were fresh and in the form of powder and then incubated for 48 hours after dipping into solvents. Further these solvents were filtered and dried for removing the traces of solvents. These dried extracts were scratch in dimethyl sulphoxide and collected into the microcentrifuge tubes for further use **[14]**.

Table 2: Collected five plant samples for the analysis of antibiofilm activity.

Botanical name	Common name	Medicinal use
Zingiber officinale	Ginger	Reduces nausea, inflammation, pain, improves digestion
Trachyspermum ammi	Ajwain	Respiratory ailments, antifungal
Morus alba	Mulberry	Diabetes, high bp, hair loss,cold, arthritis, dizziness
Murraya koenigii	Curry plant	Anti-dysentric, stomachic, skin-infections
Vitis vinifera	Grapewine	Cancer, heart disease, constipa -tion, high bp.

Antibiotic Sensitivity Test of *P.aeroginosa* against various plant samples:

Antibiotic susceptibility test is used to determine how effective antibiotic therapy is against a bacterial infection or to detect resistance in certain bacterial isolates **[15]**. This testing helps the clinicians to select the appropriate drug for the treatment of infections. The most common method of AST is Kirby-Bauer method in which small wafers containing antibiotics are placed onto a plate upon which bacteria are growing. If the bacteria are sensitive to the antibiotic, a clear ring, or zone of inhibition, is seen around the wafer indicating poor growth **[16].**

Biofilm inhibition assay of *P.aeroginosa* against screened samples by tube method:

Once biofilm of *P. aeroginosa* prepared in test tube by incubating it for 72 hours. Further the extracts were added to the tubes for analyzing the biofilm inhibition activity **[17]**.

Quantification of biofilm through ELISA plate reader:

A number of methods have been developed for cultivation and quantification of biofilms **[18]**. Basically, quantification of biofilms involves cultivation of biofilm on the walls of a test tube and subsequent detection by stain for biofilm recognition. Later, the wells of the microtitre wells are used as culture vessels

and the results are measured spectrophotometrically **[19]**. The culture medium of *Pseudomonas aeroginosa* were added to the wells and incubated. After 72 hours of incubation the plant extracts with controls were added to it and then incubated. After the completion of incubation tenure the staining of wells were carried out and then observed under microscope.

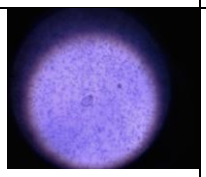
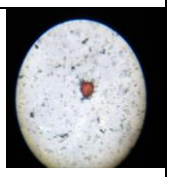
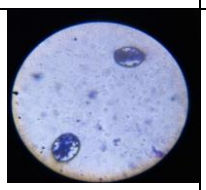
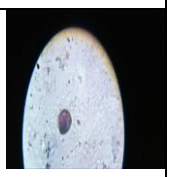
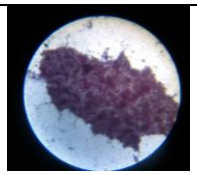
$$\% \textbf{ degradation} = 1 - \frac{(\textbf{OD Treatment})}{(\textbf{OD Control})} * 100$$

RESULTS

Microscopic analysis of biofilm formation on NB and M63 media:

The bacterial biofilms of *P. aeruginosa* were formed on coverslips and incubated 24 hrs, 48 hrs and 5 days respectively on two different media: NB and M63. The conditions for formation of biofilms were provided respectively, i.e., firstly they were kept in aerobic conditions which provided them with the necessary nutrients for growth, and secondly they were starved and kept in anaerobic conditions by transferring them to a fresh petri plates without any nutrient medium. This led to the bacteria enter into a defense mode of survival and thus form a biofilm by forming a protective EPS outer layer which protects the cells inside from outside damage. Cover slips having bacterial biofilms were visualized after staining with 0.1% crystal violet dye under 40X light microscope.

Table 3: Biofilm formation of *P. aeruginosa* on cover slips.

Duration	NB media	M63 media
24 hours		
48 hours		
120 hours		

Biofilm growth of *P. aeruginosa* by tube method

Biofilm growth by tube method is a means of qualitative assessment of biofilm formation in which test tubes containing NB media were inoculated with loop-full of microorganism from overnight culture plates and incubated for 3 days at 37°C.

The tubes were decanted and washed with PBS (pH 7.3) and dried. They were then stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with distilled water. Tubes were than dried in inverted position and observed for biofilm formation.

Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and the amount of biofilm formation was scored as 0-absent, 1-weak, 2-moderate or 3-strong.

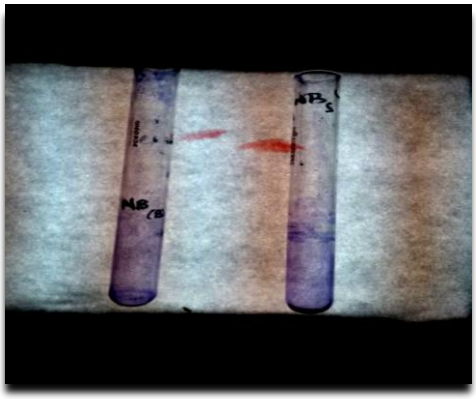


Figure 1: Biofilm of P.aeroginosa formed on test tubes and seen under white light transilluminator. Test tube marked NB (B) is taken as control without any inoculation with bacteria. Test tube marked NB(S) is the one with bacterial culture. After 3 days of incubation, a light biofilm is seen in this test tube at the air-liquid interface (seen in blue color).

Antibiotic sensitivity test against plant extracts:

AST of various plant extracts prepared in different solvents was done to determine their efficiency in inhibiting *P. aeroginosa*. The different plant extracts were examined to check the ant biofilm activity against selected bacterial strain. The screening of activity profile was done by Agar-well diffusion method; appearance of clear zone on NA plate of bacteria suggested the capability of the different extract to inhibit the growth of microbe.

The ability of a particular plant extract to inhibit the growth of pathogen was measured in terms of zone of inhibition (ZOI) calculated in mm. The extract with the maximum ZOI had the maximum ant biofilm activity when dissolved in that particular solvent. The table below shows the results given by various plant extracts:

Table 4: Antibitotic screening of plant extracts against *P. aeruginosa*

Plant sample	Zone of inhibition (in mm) against *P. aeruginosa*		
	Aqueous	Methanol	Chloroform
Ginger fresh	0	0	20.5
Ginger dry	0	29.5	24.5
Mulberry fresh	0	0	15
Mulberry dry	14	14	15
Ajwain fresh	0	0	0
Ajwain dry	0	20.5	0
Curry fresh	13	14	14.5
Curry dry	13	0	13.6
Grape wine fresh	0	0	0
Grape wine dry	17	15	15

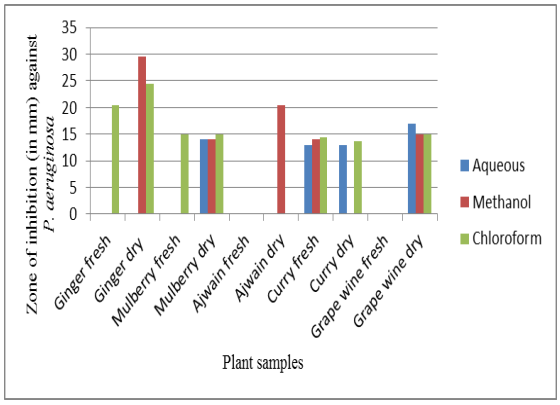


Figure 2: Graphical representation of antibitotic screening of plant extracts against *P. aeruginosa*

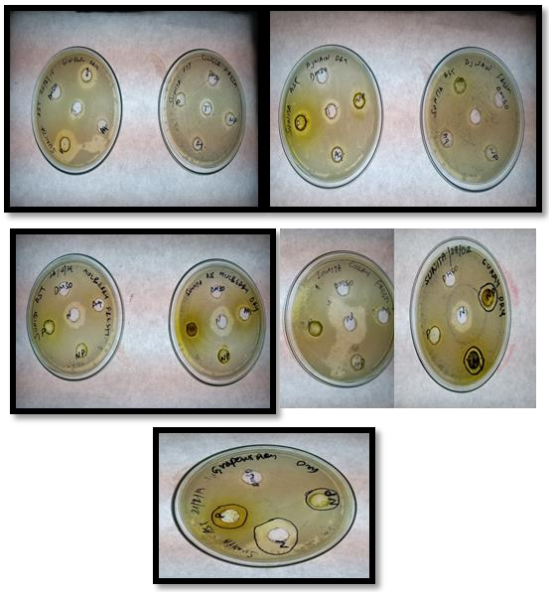


Figure 3: Antibiotic sensitivity test of Ginger, Ajwain, Mulberry, Curry and Grape wine showing zone of inhibition against *P. aeruginosa.*

Biofilm inhibition assay of *P. aeruginosa* against screened samples by tube method

Inhibition of biofilm of *P.aeroginosa* by the above screened plant samples were done in a test tube. An overnight culture of bacteria was inoculated in test tube and allowed to form biofilm, and plant extract was added at the same time. In another test tube with bacteria inoculated, crude plant extract was added after 3 days of incubation from the sides of the test tube and the results were seen after 3 days in white light.

One test tube was taken blank with no plant extract to serve as control. Inhibition of bacterial biofilm formed on the air-liquid interface shows the anti-biofilm efficiency of the plant extract.

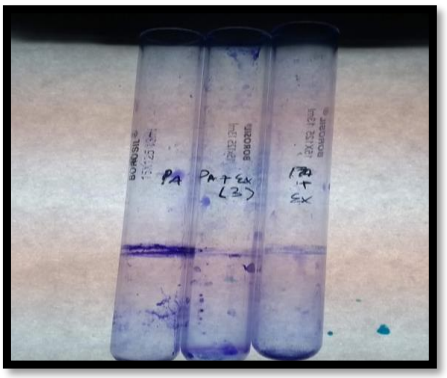


Figure 4: (L-R) Test tube marked "PA" serves as a control with high biofilm formation at the interface. Test tube marked "PA+Ex(3)" is the one in which plant sample was added at the time of inoculation and then incubated for 3 days and has less biofilm formation. Test tube marked " PA+Ex" is the one in which extract was added after 3 days of incubation and has moderate biofilm formation.

Quantification of biofilm through ELISA plate reader:

Table 5: *P. aeruginosa* % Degradation by using plant extracts

Plant extracts	***P. aeruginosa* % Degradation**
Ginger methanol (D)	96
Ginger chloroform (D)	89
Ajwain methanol (D)	52

DISCUSSION

Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years **[20].** Plant extracts, phytochemical compounds and essential oils have been investigated for their ability to treat or prevent adhesion of oral bacteria to various surfaces. The inhibitory effects of natural compounds on biofilm forming oral bacteria have also been tested.

Plants like arak has been reported to have many pharmacological properties on cariogenic and periodontal pathogens such as *S. mutans, Streptococcus salivarius, Staphylococcus aureus* **[21].** The ability of cranberry juice or cranberry extracts on *P. gingivalis, S. mutans* and other oral *streptococci* to prevent adhesion of oral pathogens has been widely investigated **[22]**.

Alcoholic extract of *M. alba* leaves has shown reduction of the glucosyltransferase activity in terms of glycan production, reduction in the bacterial density of the biofilm and the decrease in thickness of the preformed biofilm of *S. mutans.* In vitro evaluation of the anti-cariogenic potential against *S. mutans* concluded a significant effect of ajwain seeds on dental caries inhibition by reducing the biofilm-forming tendency. Furthermore, mulberry leaves were reported to have a grape marc extract was able to affect the formation of a *S. mutans* biofilm and to significantly reduce the glucotransferase B (GTF) activity in a dose-dependent manner **[23].** The essential oils of eucalyptus and rosemary are used for flavoring, perfume production, and medicinal purposes.

Recently, several potential quorum sensing inhibitory (QSI) have been discovered from various resources (Kalia, 2013). *Curcuma longa* (turmeric) produces curcumin, which inhibits the expression of virulence genes of *P. aeruginosa.* Furthermore, extracts from different plant parts such as the leaves, flowers, fruit, and bark of *Laurus nobilis* (bay leaves) and *Sonchus oleraceus* (milk thistle) were also found to possess anti-QS activities **[24]**.

In the present study, efforts have been put to inhibit the biofilm caused by *Pseudomonas aeruginosa*, which is the leading cause for nosocomical infections and infections like Cystic fibrosis, Wounds Eye infections, urinary tract infections and Cardiac valves (endocarditis). Primary development of *P.aeroginosa* biofilm on a cover slip and its visualization under a microscope led us to the understanding of the shape and colonization of these bacteria, as well as the conditions (time, temperature) required for it to develop. Continuing with the work of previous researchers on their efforts to test the antibiofilm activity of various plants on *S. mutans,* in our study we have taken 5 plant samples and tested their efficacy in inhibiting the growth of *P. aeroginosa* biofilm. These plant samples were prepared in different solvents and a total of 30 extracts were tested. The most promising results of Antibiotic Sensitivity Test were shown by ginger plant extract dissolved in polar methanolic solvent. Ajwain also showed good results. These plants were screened in to test their efficiency in inhibiting fungal strains of *A. niger* and *C. albicans* that also form biofilms

Ginger was able to inhibit *C. albicans* strains up to a certain extent when AST was performed.

CONCLUSION

Scientific interest in biofilms has exploded in the past decade. This fascination with biofilms is due to their clinical relevance. But it is also due to the appeal of projecting traits of higher organisms on these life forms that were once thought to be so simple. The ability of prokaryotes to adapt to their surroundings is remarkable, but whether they actually communicate, coordinate, and specialize within biofilms for the benefit of the community, as opposed to simply reacting to their environments in order to selfishly promote their own survival, has not yet been sufficiently established. Thus, we can conclude that certain questions are purely hypothetical and cannot be tested scientifically. Such questions can be objectively examined, and scientists have recently begun to develop mathematical models that predict the relative impacts of altruistic vs. selfish behavior on the survival and propagation of bacteria. Our path seems clear. We have come to understand many things about the unique biology of bacterial biofilms. Biofilms represent microbial societies with their own defense and communication systems. We have an arsenal of microscopic, physical chemical and molecular techniques available to examine biofilms. There are many basic questions regarding the biology of biofilms that can now be answered. Our modern view of biofilm infections leads to the realization that their effective control will require a concerted effort to develop therapeutic agents that target the biofilm phenotype and community signaling–based agents that prevent the formation, or promote the detachment, of biofilms. The techniques are now available to undertake such efforts.

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