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Eradication of Nosocomial biofilm using novel quorum sensing inhibitors against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Julbihar Ahamed K, Kulandhaivel M, Hridhya KV
Department of Microbiology, Karpagam University, Coimbatore-641 021, Tamil Nadu, India
Email: kulandhaiveljm@gmail.com

ABSTRACT

Biofilm is a group of microorganisms inin which microbial cells adhere to each other on a living or non-living surfaces within a self-produced matrix of extracellular polymeric substance. Bacterial biofilm is infectious in nature and can results in nosocomial infections.It has been found bacterial biofilm mode of growth are often up to 1000 times more resistance to antibiotic than the vegetative form of growth. Quorum sensing is a regulatory mechanism; it can alter bacteria to make mass selection with respect to the expression of a specific set of genes. It release and subsequent detection of chemical signaling molecules, such as N-acylhomoserinelactones (AHLs). When the concentration of AHLs reaches a certain threshold level, binding to a receptor molecule (for example, LuxR) is promoted and the activated LuxR–AHL complex forms dimers or polymers, which, in turn, act as transcriptional regulators of target genes in the Quorum Sensing regulon . Quorum Sensing Inhibitors have been proposed as promising antibiofilm agents. Quorum Sensing Inhibitors which have been shown to play a role in biofilm formation and/or maturation. As plants have co-existed with Quorum Sensing bacteria for millions of years, it can be expected that at least some of them produce Quorum Sensing Inhibitors in order to reduce the pathogenic capability of infective bacteria. The compounds were separated by Ethanolic extract of Ocimum sanctum and Ethanolic extract of Seasamum alatum Thonn was separate and purified in column chromatography. Halogenated furanone C30 was used as an standard Quorum Sensing Inhibitor.
Keywords: Nosocomial biofilm, Sensing Inhibitor, AHLs

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INTRODUCTION

Biofilm is a group of microorganisms in which microbial cells adhere to each other on a living or non-living surfaces within a self-produced matrix of extracellular polymeric substance. Bacterial biofilm is infectious in nature and can results in nosocomial infections.

Nosocomial infections are the major leading cause of death in many countries, and about 65% of these infections are due to biofilmon implanted medical devices. This is due to the property of Extra Poly Saccharide (EPS) matrix of the biofilm. This EPS matrix also keeps the normal functions of antibodies and the phagocytic cells of the host's immune system. Another key factor that makes bio films particularly difficult in treatment is their heightened resistance to antibiotics. It has been found that bacterial biofilm mode of growth are often up to 1000 times more resistance to antibiotic than the vegetative form of growth [1].

The antibiotic may not be able to penetrate the surface layers of the bio film, or it is deactivated faster than it diffused. The action of the antibiotic can produce different chemical environments throughout the biofilm[14]. Decreased antibiotic susceptibility contributes to the durability of biofilm infections such as those connected with implanted devices. In the biofilm possibly to comprise a multi level defence system because of the poor antibiotic penetration properties, slow growth, nutrient limitation and adaptive stress responses are observed. The biochemical and genetic information inside of these biofilm defences are the current scenario.

Quorum sensing is a regulatory mechanism; it can alter bacteria to make mass selection with respect to the expression of a specific set of genes. The key role is release and subsequent detection of chemical

signaling molecules, such as N-acylhomoserine lactones (AHLs). When the concentration of AHLs reaches a certain threshold level, binding to a receptor molecule (for example, LuxR) was promoted and the activated LuxR–AHL complex forms dimers or polymers, which, in turn, act as transcriptional regulators of target genes in the QS regulon [10]. The Quorum Sensing systems of *P. aeruginosa* are also involved in raising tolerance to antibiotic as well as tolerance to the activity of host immune systems [1, 3].

The Biofilmformation begins with the adhesion of a small amount of bacterial cells to a surface. Most of the laboratory-adapted strains have lost their ability to adhere to surfaces. Whenever cultures are transferred into fresh medium, the inoculums for this operation is taken from the bulk fluid and bacterial cells that are adherent to the walls of the vessel are left behind. After a few hundreds of transfers of this type of adapted bacterial strains have been selected for rapid growth proliferation.

Pseudomonas aeruginosa is a gram negative, rod shaped, motile opportunistic pathogenic bacterium which is responsible for both acute and chronic infections. *P.aeruginosa* by nature shows resistance to many drugs, its ability to form biofilm requires a complex biological system, renders ineffectual the clearance by immune defence systems. *Pseudomonas species* was one of the most important biofilm species. Bacterial biofilm are often involved in chronic infections, where they can elicit revenant and persisting pathologies. Bacteria living in Biofilm are less susceptible to the inflammatory and immune responses of their host and are considerably more resistant to antibiotic treatment than planktonic bacteria and accordingly, are more difficult to eradicate. Most of the *P.aeruginosa* produces at least three polysaccharides (Alginate, Pel, and Psl) that are a factor for the stability of the biofilm structure. The qualitative composition of the polysaccharides differs from the mucoid and nonmucoid. *P. aeruginosa* strains. Alginate is a linear unbranched polymer, it composed of D-mannuronic acid and L-guluronic acid. These compounds contribute to the stability and protection of biofilms. The Pel polysaccharide is a glucose-rich matrix material, with still unclarified composition. Psl is combined of a pentasaccharide-repeating unit of D-mannose, D-glucose and L-rhamnose.Psl acts as an adhesin and plays a crucial role in the formation and maintenance of the biofilm architecture in *P. aeruginosa* infections. Pel and Psl are involved at initial stages of biofilm formation [8].

Staphylococcus aureus is a Gram positive, Cocci, non motile ubiquitous bacterial species. There is a strong causal connection between *S. aureus* nasal carriage and increased risk of nosocomial infection. Nasal carriage provides a staging ground for *S. aureus* to distribute to other areas of the body where, once transmitted to the circulatory system through an epithelial breach, planktonic growth and up regulation of adherence factors occurs [9]. Invading *Staphylococci* are then either removed by the host innate immune response or attach to host extracellular matrix proteins and form a biofilm. *Staphylococcus aureus* can produce a multi-layered biofilm attached within a glycocalyx or slime layer with heterogeneous protein expression throughout. *Staphylococcus aureus* has re-emerged as a clinically relevant pathogen due to its resistance to antibiotics and the increased use of indwelling medical devices.

Quorum Sensing Inhibitors (QSI) has been proposed as promising antibiofilm agents. bQuorum Sensing Inhibitors which have been shown to play a role in biofilm formation and/or maturation [2].As plants has co-existed with QS bacteria for millions of years, it can be expected that at least some of them produce QSIs in order to reduce the pathogenic capability of infective bacteria. Some plants have been used for thousands of years in Traditional Medicines [7]. Since ancient times, plant extracts and phytochemicals have been often used in nutrition and medicine because of their wide range of beneficial effects against chronic diseases as well as their antimicrobial activities.

In fact, crude plant extracts are found to be more effective than their isolated constituents at an equivalent dose attributing to positive interactions between components of the whole plant extracts. Hence, it is lately fulfilled that crude extracts are possibly the right strategy to treat the multi-drug resistant pathogenic bacteria comparing to the purified compounds isolated from the same extract.

The chemical composition of *Ocimum sanctum* is highly complex, containing numerous biologically active compounds.The pharmacological properties of the entire plant in its natural form, as it has been traditionally used, result from synergistic interactions of many different active phytochemicals. Consequently, the overall effects of *O.sanctum* cannot be fully duplicated with isolated compounds or extracts. Because of its inherent botanical and biochemical complexity, *O.sanctum* standardization has, so far, eluded modern science [12].

Sesamum alatum is widely spread in tropical Africa, occurring in dry regions from Senegal to South Africa. In Madagascar, India and occasionally elsewhere it has been introduced. The leaves of *S. alatum* are used as an aphrodisiac and to cure diarrhoea and other intestinal disorders.

Owing to the multi-component mechanisms used for plant associated bacterial control and inhibition of pathogenic bacteria, further studies on the purification of active components and production of medicinal plant activities based on novel strategies are vital [4]. Moreover, a detailed chemistry of the active

compounds and further evaluation of their mechanisms of actions and interaction with microbial processes are needed to be addressed.

MATERIAL AND METHODS

Bacterial Isolates

Pseudomonas aeruginosa and *Staphylococcus aureus* was obtained from the Karpagam Medical College Hospital, Coimbatore.

Bacterial Identification: Identification of bacterial cultures was done by using following methods using manual [5, 17, 18]. The cultures were preliminarily identified based on morphological characteristics like colour, size, margin, form, elevation and texture.

The gram staining, Motility Staining and endospore staining prepared for bacterial identification. Biochemical tests are the tests used for the identification of bacteria species based on the differences in the biochemical activities of different bacteria.

Compounds

The compounds were separated by Ethanolic extract of *Ocimum sanctum* and Ethanolic extract of *Seasamum alatum Thonn* [15]was separated and purified in column chromatography. Halogenated furanone C30 was used as an standard Quorum Sensing Inhibitor. Qualitative and quantitative analysis of phytochemicals study of the plant extract were performed [6].

Biofilm Formation

Method 1

The mass Biofilm was measured by the modification technique was performed. The 1ml of bacterial cells were mixed with the 200 µM of different compound and 1% (v/v) glycerol in 2ml microfuge tubes. Then the tubes are incubated at 37° for 72 hours. The cultures in tubes were removed and the tubes were rinse with double distilled water for 3 times to remove loosely associated bacteria. Eventually, the tubes were air dried. Then each dried tube was stained with 1 ml 0.5% (w/v) crystal violet solution for half an hour and washed three times with double distilled water to remove excess amount crystal violet. The quantitative analysis of biofilm was performed by adding 1 ml 95% (v/v) ethanol into the tube for 15 min. Then, the crystal violet present in the ethanol solution was measured by spectrometry at 570 nm.

Method 2

This technique was modification method of O'Toole and Kolter technique. The *P. aeruginosa* colony was inoculated in Cetrimide Broth (Purchased from Hi Media, Mumbai) and the *S. aureus* colony was inoculated in Mannitol Salt Broth (Purchased from Hi Media, Mumbai). Both are incubated at 37°C for 18 h. It was then diluted with fresh broth and the turbidity was adjusted to 0.5 McFarland standards. 200 µL of suspension was dispensed into the microtiter plate wells in duplicate. The plates were incubated 37°C for 24 h. The contents were aspirated and washed with Phosphate Buffered Saline (pH 7.4). Then 100 µl Bouin Fixative agents were added. The plate was incubated at Room Temperature for 10 min. The contents were discarded, and the wells were stained with 1% crystal violet. After 1 min, the excess stain was rinsed off by placing the plate under running tap water. Then, 33% glacial acetic acid was added to each well and optical densities of stained adherent bacterial films were read with Micro ELISA plate reader at 570nm.

Statistical analysis

All experiments were performed in duplicate. Statistical analysis was performed using SPSS 17.0 for Windows (SPSS, Inc., Chicago, IL, USA).

RESULT

Bacterial Isolates

Pseudomonas aeruginosa

Pseudomonas aeruginosa was identified as Gram Negative, Rod Shaped, Motile with Unipolar Flagella, Non Capsulated, and Non Spore Forming Bacteria. The Biochemical results are Indole Negative, Methyle Red Negative, Voges–Proskauer Negative, Citrate Positive, Urease Negative. It produces acid, Ferment Mannitol.

Staphylococcus aureus: Staphylococcus aureus is identified as Gram Positive, Cocci, Non Capsulated, and Non Spore Forming Bacteria. The Biochemical results are Indole Negative, Methyle Red Negative, Voges–Proskauer Positive, Citrate Positive, Catalase Positive and Hemolysis on blood agar in beta hemolysis. It produce acid but no gas, Ferment Mannitol, Glucose, Fructose and Maltose.

Biofilm Formation

The result was observed under the UV Spectrophotometer at 570 nm and tabulated.

Table 1. Halogenated Furonane c30 is an standard QSI for this study. Sample 1 and 2 is derived from *Ocimum sanctum*, Sample 3 and 4 derived from *Seasamumalatum Thonn* and Sample 5 is Synthesizes Silver Nanoparticles.

Sample	OD Value for (*S. aureus*)	OD Value for (*P. aeroginosa*)
Control 1	3.191	-
Control 2	-	2.058
Sample 1	1.850	1.544
Sample 2	2.589	2.000
Sample 3	0.707	0.592
Sample 4	0.698	0.416
Standard	0.812	0.793

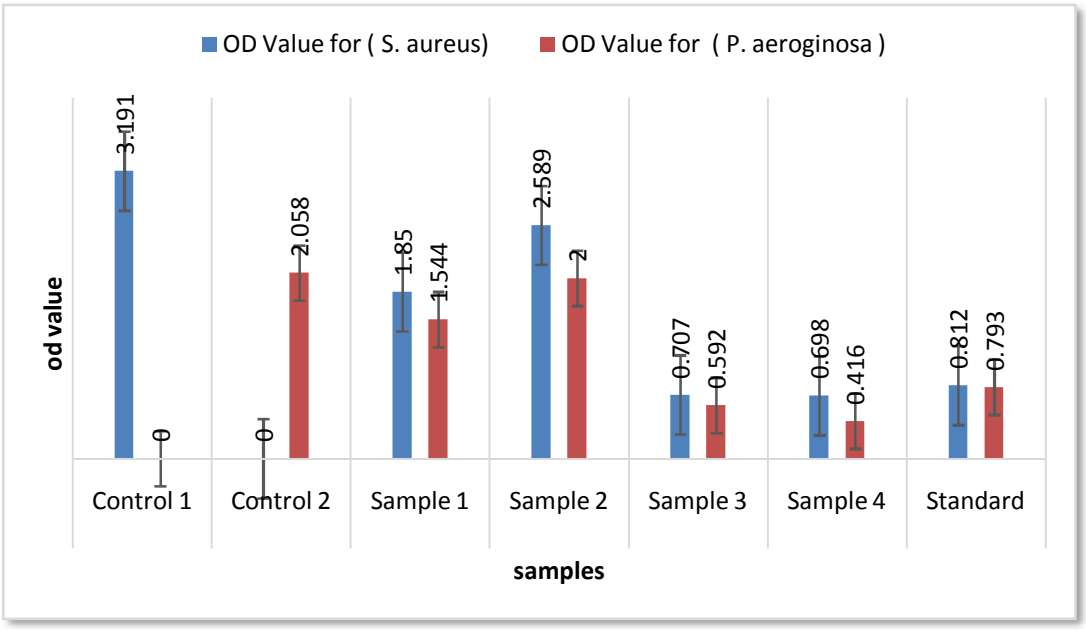

Fig 1. Comparison of biofilm eradication on *S. aureus* and *P. aeruginosa*

Method 2:

The result was observed on the ELISA plate reader on 570 nm. The sample was processed with two different concentrations and tabulated.

Table 2. Halogenated Furonane c30 is an standard QSI for this study. Sample 1 and 2 is derived from *Ocimum sanctum*, Sample 3 and 4 derived from *SeasamumalatumThonn* and Sample 5 is Synthesizes Silver Nanoparticles.

Organisms	*Pseudomonas aeruginosa*		*Staphylococcus aureus*	
Concentration	**1 X**	**0.5 X**	**1 X**	**0.5 X**
Control 1	1.375	1.375	-	-
Control 2	-	-	1.693	1.693
Sample 1	1.042	0.340	0.468	0.158
Sample 2	1.161	0.483	1.088	0.432
Sample 3	1.102	0.524	0.873	0.567
Sample 4	0.305	0.273	0.427	0.238
Standard	0.696	0.696	0.890	0.890

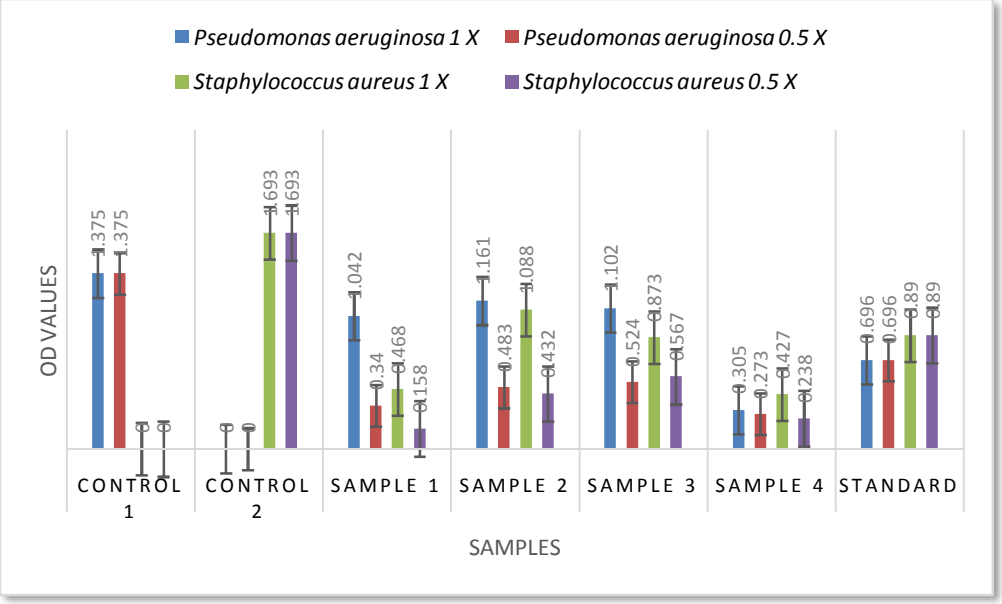

Fig 2.Comparative studies of biofilm eradication on *S. aureus* and *P. aeruginosa* in different concentration.

DISCUSSION

The Eradication study of the nosocomial biofilm using quorum sensing inhibitor analyzed by two different techniques. In the first technique of You et al was done with five samples. The First two sample (Sample 1 and Sample 2) was derived from the ethyle acetate extract of *Ocimum sanctum*. The Sample 3 and Sample 4 were derived from the ethanolic extract of *Seasamum alatum thonn.* The sample 3 and Sample 4 has better eradication values than others followed by sample 1 is moderate eradication values. In both pathogens. The sample 3 eradicate 77.8% of biofilm in *Staphylococcus aureus* and eradicate 71.23% in *Pseudomonas aeruginosa*. The sample 4 eradicate 78.1% of biofilm in *Staphylococcus aureus* and eradicate 79.78% in *Pseudomonas aeruginosa*. The sample 1 eradicate 42.02% of biofilm in *Staphylococcus aureus* but eradicate only 24.97% in *Pseudomonas aeruginosa*.

In second method modified method of O'Toole and Kolter technique, the eradication study was done with five samples. In this study was determined by the two different concentrations. That is 1X and 0.5X concentration. The *Pseudomonas aeruginosa* in 1X concentration sample 4 (77.8%) better eradication ratio followed by sample 1(24.2%). But in 0.5 X all samples are better eradication ratios. In *Staphylococcus aureus* both 1X and 0.5 X are eradicate the better values. This result shows the concentration was very important factors in the quorum sensing inhibitors. *Pseudomonas aeruginosa* biofilms are easily eradicated by tobramycin when they are pretreated with QSIs [13]. An effective and applicable QSI should require these properties[13]: [1] the QSI is a low-molecular-mass molecule the activity of which causes a significant reduction in the expression of QS-controlled genes, [2] the inhibitor exhibits a high degree of specificity for the QS regulator (i.e., the LuxR homolog) without side effects on either the bacteria or an eventual eukaryotic host, [3] the QSI should be chemically stable and resistant to metabolism and disposal by the higher host organism. Baicalein meets these requirements.

CONCLUSION

Results of the present eradication study demonstrated the happening of resistance to various antibacterial agents along with the bacterial cell isolates. In clinical study Pseudomonal and Staphylococcus biofilm was grown easily when compared to other pathogenic organism. In now days Nosocomial strains was easily resist to the antimicrobial drugs because of frequent usage of antibiotics.

Besides this, the organisms also produce biofilms which serve as barriers to effective therapy. Regular antimicrobial susceptibility monitoring would help and guide the physicians in prescribing the right combinations of antimicrobial to limit and prevent the emergence of multi drug resistant strains of Bacteria. Antibiotics should be used judiciously and at the optimum concentration so as to inhibit biofilm formation and eradicate persisted cells. As this is a hospital-based epidemiological data, the present study will help in implementation of better patient management and infection control strategies. The need to develop new anti-pathogenic drugs is especially acute in the case of *P. aeruginosa* and *Staphylococcus* infections in cystic fibrosis patients, where the natural antibiotic resistance of the bacteria and the ability

to form biofilms account for significant mortality in such patients [11]. Therefore, baicalein described in the report provides the potential for a new class of antibacterial agents to target infections that are persistently difficult to combat with the current antibacterial agents.

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