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Inhibition of Antibiofilm Mediated Virulence Factors Production in Urinary Pathogen *Serratia marcescens* by *Vitex Trifolia*

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Abstract: ***Quorum sensing (QS) is well known for bacterial virulence, antibiotic resistance and biofilm production. Inhibition of quorum sensing can reduce the risk of microbial pathogenicity in all infection. In this present study the vitex trifolia leaf extract was assessed for its anti quorum sensing activity against acyl homoserine lactone (AHL)-dependent biofilm formation in Serratia marcescens. The methanol extract obtained from Vitex trifolia exhibited a concentration dependent (50-100 µg/ml) reduction in biofilm production in Serratia marcescens to a level of 50-80%. Microtiter plate and Light microscopic analysis further confirmed the reduction in the biofilm forming ability of Serratia marcescens when treated with this extract. In addition the Vitex trifolia extract by itself showed no growth inhibitory effect on S. marcescens.***

Keywords: *Vitex trifolia*, Quorum sensing inhibition, Acyl homoserine lactone, *Serratia marcescens*, anti bacterial assay, Biofilm

1. Introduction

Several environmental stresses have been demonstrated to increase polysaccharide intercellular adhesion (PIA) synthesis and biofilm formation by the human pathogens *Serratia marcescenes*. In this study we characterized an adaptive response of *Serratia marcescenes* to *Vitex trifolia* induced stress and show that it involves concomitant impairment of PIA synthesis and biofilm formation. *Serratia marcescens* is gram negative bacterium and it is an opportunistic human pathogen causing several infections. *Serratia marcescens* causes infections including respiratory tract, urinary tract, meningitis, septicemia, pneumonia and wound infections by secreting a number of virulence factors capable of damaging human cells and tissues [2]. It shows high resistance against antibiotic. The wide range antibiotics which include b-lactam, aminoglycoside and fluoroquinolone treatment become ineffective [10]. Thus, the emergence of antibiotic resistance among bacterial pathogen necessitate for the findings of alternative strategies to antibiotic treatment. It has been known that in S. marcescens the quorum sensing (QS) regulates the expression of genes responsible for the production of prodigiosin pigment, virulence factors like hemolysin, proteases, chitinase, chloroperoxidase, multiple isozymes of alkaline phosphatase, the ability to swim, swarm and biofilm maturation [6,7].

Vitex trifolia was considered to be a rich depository of bioactive compounds with antibacterial, antifungal, antiviral, antifouling, anti-HIV, antibiofilm, immunosuppressant and cytotoxic activities. *Vitex trifolia* are known to produce powerful anti-biofilm and anti-inflammatory compounds [3]. Similarly, the methanol extract of *Vitex trifolia* have shown a strong quorum sensing inhibitory (QSI) activity and acted as a good antagonist against the QS systems of *S. marcescens* [8]. Although, they are known for their bioactive potential, studies on its QSI properties remain scanty. Hence, the present investigation is aimed to study the effect of QSI activity of *Vitex trifolia* extracts against the QS systems of *S. marcescens.*

2. Materials and Methods

2.1 Preparation of *Vitex trifolia* leaf Extracts

Vitex trifolia leaves used in this study were collected from well grown trees in Tamilnadu Agriculture University, Katuthotam, Thanjavur district, Tamilnadu. The leaves were washed twice with ordinary water followed by distilled water. The washed leaves were shade dried and powered using blender. 5gm of powered sample were soaked in 50 ml of methanol for overnight. The methanol phase was collected and dried at 55°C. The residues were collected and redissolved with deionized water. Finally stored at -20°C for further use.

2.2 Bacterial Strains and Their Culture Conditions

Serratia marcescens used in this study is a clinical strain isolated from a patient with urinary tract infection at kings institute of preventive medicine, Guindy, Chennai. The isolated strain was identified through 16S rRNA gene sequences (Gen- Bank accession number: FJ584421). The bacterial strain was allowed to grow aerobically in Luria–Bertani slants. From the slants, sub culture were done and cultures were maintained in (LB) broth (Hi-Media, India) at an optimum temperature (30° C) and intensity was checked using UV spectrometer at OD 600nm before using for further analysis.

2.3 Antibacterial assay

Antibacterial activity of the *Vitex trifolia* leaf extracts with QSI potential was performed in Muller–Hinton agar (MHA) (Hi Media, India) by the method followed by the Clinical and Laboratory Standards Institute (2006). The 100 µl of test bacterial suspensions which were expected to have cell

density equivalent to 0.5 McFarland standards (approximately 1×108 CFU/ml) were uniformly spread over the surface of the MHA plate. Then, the sterile paper disks (Hi Media, India) with a diameter of 10 mm loaded with various concentrations (50–100 µg) of *Vitex trifolia* extracts were placed over the plates and incubated at 30 °C for 24 h and observed for growth inhibition zone[5].

2.4 Growth curve analysis

One percentage of overnight culture of bacterial pathogens (0.4 OD at 600 nm) were inoculated in 250 ml Erlenmeyer flask containing 100 ml of LB broth supplemented with various concentrations (50-100 µg) of *Vitex trifolia* extract. The flasks were incubated at the optimum temperature which is suitable for *Serratia marcescens* under 180 rpm in a rotatory shaker. The cell density was measured in UV–visible spectrophotometer at every one hour interval [4].

2.5 Biofilm formation in 24-well MTP

The effect of *Vitex trifolia* extract on the biofilm formation on *Serratia marcescens* was determined by quantifying the biofilm biomass through Micro Titer Plate assay [9]. Briefly, 1% of overnight cultures with OD adjusted to 0.4 at 600 nm. The test pathogen were added into 1 ml of fresh LB medium and cultivated in the presence and absence of *Vitex trifolia* extract (50-100 µg/ml) without agitation for 16 h at 30 °C. After 16 h incubation, the planktonic cells in MTPs were removed by rinsing the wells. The wells were rinsed twice with sterile distilled water. The surface-adhered cells in the MTP wells were stained with 250 µl of 0.2% crystal violet (CV) solution (Hi Media, India). The solutions were left in MTP wells for 10 mins. Then the excess CV solution was removed. So, CV in the stained cells was solubilized with 1 ml of 95% ethanol. The biofilm biomass was quantified by measuring the intensity of CV solution. The intensity was measured at OD 650 nm using UV–visible spectrophotometer.

2.6 Biofilm Prevention Assay

Biofilm prevention assay was done by adding 10 µl of over night culture of S. marcescens in above mentioned cell density to 1 ml of LB broth in 24 well MTP containing glass slides (1×1 cm) and supplemented with and without and *Vitex trifolia* extracts (50-100 µg/ml). Culture set up was incubated without agitation at 30°C for 18 h [9]. After the incubation, planktonic cells and media were discarded. The adherent cells which sticked in glass slides were gently rinsed twice with deionized water (Millipore–Milli-Q) [1].

2.7 Light microscopic analysis

One percentage of overnight *Serratia marcescens* cultures (0.4 OD at 600 nm) were added into 1 ml of fresh LB medium which containing cover glass of 1 cm^2 along with and without *Vitex trifolia* extracts (50-100 µg/ml). After 16 h of incubation, the cover glasses were rinsed thrice by using distilled water to remove the planktonic cells and biofilms. Then the cover glasses were stained with 0.2% CV solution. Stained cover glasses were placed on slides. The biofilm were pointed up and visible biofilms were visualized by light microscope at magnifications of 40X (Olympus CK ×41 Jenoptik Germany, ProRes C5) [9].

3. Results and Discussion

Biofilms are a highly dense matrix-encapsulated population which was attached to the surfaces [9]. The biofilm formation in *S. marcescens* is a major virulence factor which is controlled by *Vitex trifolia*. Biofilm has the ability to resist host immune response. It also resists conventional antibiotics. So, the control measures are required to prevent the biofilm formation in bacterial cells [9]. In the present study, biofilm images revealed that the *Vitex trifolia* extracts effectively disturb the biofilm formation as shown in light microscopic analysis. In this study *Serratia marcescens* was used as target pathogenic model to know anti-biofilm activity of *Vitex trifolia*. The influence of methanol extract of *Vitex trifolia* was assessed for its ability to inhibit biofilm formation in *S. marcescens*. The minimum inhibition of biofilm was 50µg/ml and maximum inhibition of biofilm was 100µg/ml. The order to analyze the antibiofilm efficiency of *Vitex trifolia* extract in inhibiting biofilm formation, *S. marcescens* cells were allowed to grow in MTP having glass slide in presence and absence of *Vitex trifolia* extract and the results were identified and visualized under a light microscope (Fig.1 & 2).

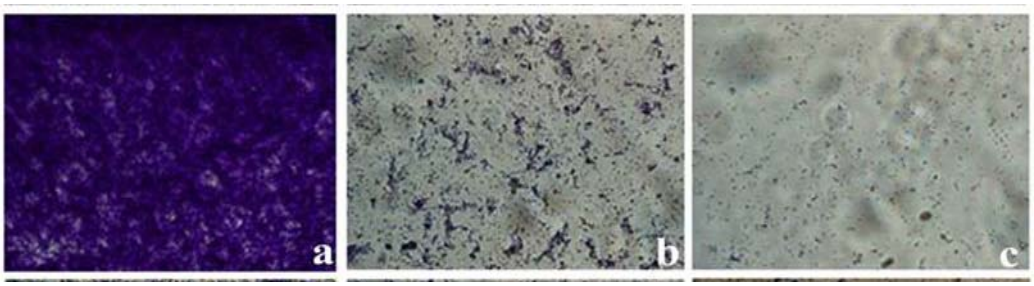

Figure 1: Light microscopic view of *Serratia marcescens* treated with *Vitex trifolia*.
a. untreated control, b. treated with 50µg of extract, c. treated with 100µg of extract.

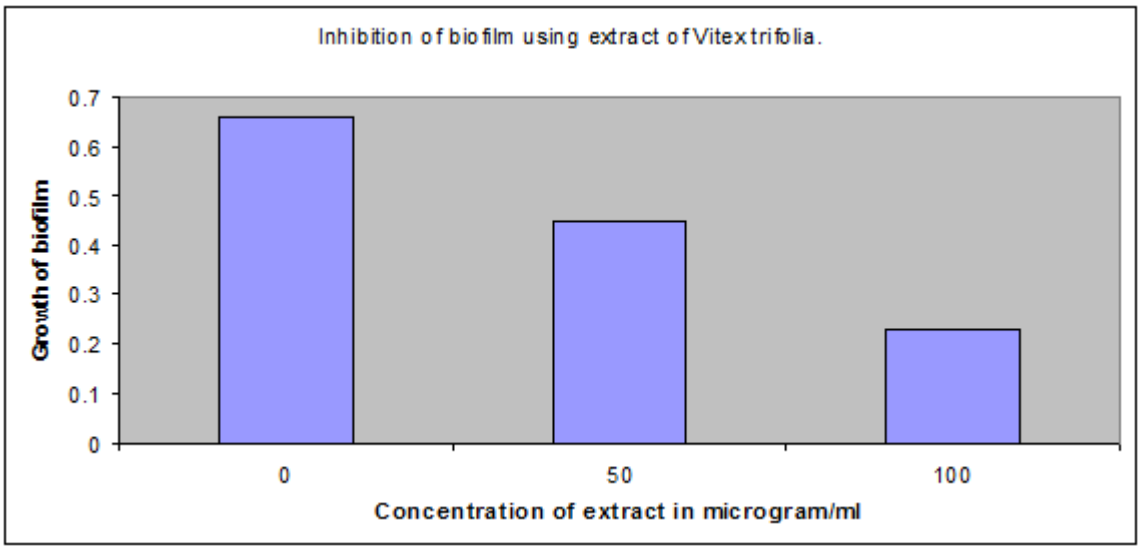

Figure 2: Graphical representation of biofilm inhibition in *S. marcescens* using *Vitex trifolia.*

4. Conclusion

In the present study, since the *Vitex trifolia* extract were made by using methanol, the possibility of anti-biofilm activity was been ruled out. Therefore, it is envisaged that the active principle exhibiting QSI activity might be an analogue of AHL molecule. However, the extract needs further purification and characterization to find out the active principle with the antibiofilm inhibitor activity. In conclusion, the reduction in QS controlled expressions and the end effect on virulence factors production without affecting the bacterial growth provided some insight into the potential of *Vitex trifolia* as QS inhibitors. Therefore, it is envisaged that the QSI potential of these *Vitex trifolia* could be used as anti-biofilm drug to combat with *S. marcescens* and also with other bacterial infections.

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