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Original Article

SCREENING OF ANTIBIOFILM AND ANTI-QUORUM SENSING POTENTIAL OF *VITEX TRIFOLIA* IN *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Objective: In this present study the methanolic leaf extract of *Vitex trifolia* was assessed for its anti quorum sensing activity against acyl-homoserine lactone (AHL)-dependent virulence factors in *Pseudomonas aeruginosa.*

Methods: In this study the methanolic leaf extract of *Vitex trifolia* was screened to control the virulence factors of *Pseudomonas aeruginosa* by antibacterial analysis, biofilm prevention assay, protease and elastase assay, pyocyanin production, EPS (Exopolysaccaride) assay, Swimming assay and Light microscopic analysis.

Results: The methanolic leaf extract obtained from *Vitex trifolia* exhibited a concentration dependent (50-100 µg/ml) reduction in anti quorum sensing activity in *Pseudomonas aeruginosa*. The extract inhibited 69% of protease activity, 48% of elastase activity and 96% of exopolysaccharide production in *Pseudomonas aeruginosa*. The pigment pyocyanin production and swimming motility of the bacteria were also controlled.

Conclusion: Thus in this study the methanolic leaf extract of *Vitex trifolia* was found to be potent against anti quorum sensing activity in *Pseudomonas aeruginosa*.

Keywords: *Vitex trifolia*, Quorum sensing inhibition, Acyl homoserine lactone, *Pseudomonas aeruginosa*, Antibacterial assay, Antibiofilm.

INTRODUCTION

Vitex trifolia is known to possess various active constituents such as halimane-type diterpenes, essential oils, vitetrifolins and several pharmacological properties have been studied viz., antipyretic, and antibacterial, against asthma, allergic diseases and also used for treating acute jaundice. *Pseudomonas aeruginosa* is a gram-negative bacterium and it is an opportunistic human pathogen causing several infections. *Pseudomonas aeruginosa* is a common bacterium that can cause disease in animals, including humans. It is citrate, catalase, and oxidase positive. Several environmental stresses have been demonstrated to increase polysaccharide intercellular adhesion (PIA) synthesis and biofilm formation by the human pathogens *Pseudomonas aeruginosa*. It is found in soil, water, skin flora, and most man-made environments throughout the world. It thrives not only in normal atmospheres, but also in hypoxic atmospheres, and thus has colonized many natural and artificial environments. It uses a wide range of organic material for food. In animals, its versatility enables the organism to infect damaged tissues or those with reduced immunity. The symptoms of such infections are generalized inflammation and sepsis. If such colonizations occur in critical body organs, such as the lungs, the urinary tract, and kidneys, the results can be fatal [1].

This bacterium is the leading cause of life-threatening infectious diseases, including urinary tract infections, respiratory tract infections, dermatitis, chronic wounds, and severe burn wound infection, and most importantly, it causes cystic fibrosis in immunocompromised patients [2, 4]. It thrives on moist surfaces; this bacterium is also found on and in medical equipment, causing cross-infections in hospitals and clinics. It is also able to decompose hydrocarbons and has been used to break down tar balls and oil from oil spills. *Pseudomonas aeruginosa* is an opportunistic human pathogen, listed as the second most common gram-negative bacterial pathogen by the National Nosocomial Infection Surveillance System [1]. Conventionally, an antibiotic therapy has been used to treat infections caused by *Pseudomonas aeruginosa* [2]. However, the subsequent development of antibiotic resistance of *Pseudomonas aeruginosa* to the available antibiotics has also been well documented [5, 6]. Therefore, there is a pressing need to find alternative treatment measures to prevent emerging *Pseudomonas aeruginosa* infections [7]. In this study, we characterized an adaptive response of *Pseudomonas aeruginosa* to *Vitex trifolia* induced stress and shown that it involves concomitant impairment of polysaccharide intercellular adhesion (PIA) synthesis and biofilm formation including anti quorum sensing response.

MATERIALS AND METHODS

Preparation of *Vitex trifolia* leaf extracts

Fresh leaves of *Vitex trifolia* used in the present study were collected from Tamilnadu Agriculture University, Katuthotam, Thanjavur district, Tamilnadu. The leaves were washed with ordinary water followed by distilled water. The washed leaves were shade dried and powered. 10 gm of powered sample were soaked in 100 ml of methanol for overnight. The methanol phase was collected and dried at 55 °C. The residues were collected from the dried methanolic phase and redissolved with deionized water. Finally, the concentrated sample was stored at-20 °C for further use.

Bacterial strains and their culture conditions

The test pathogen, *Pseudomonas aeruginosa* used in this study, was obtained from King Institute of Preventive Medicine, Guindy, Chennai, which was isolated from the patient with skin and soft tissue infection. The bacterial strain was allowed to grow aerobically in Luria–Bertani slants. Subculture was done from the aforementioned slants and cultures were maintained in (LB) broth (Hi-Media, India) at an optimum temperature (37 °C) [6].

Antibacterial assay

Antibacterial activity of the *Vitex trifolia* leaf extracts with Quorum Sensing Inhibitory potential was performed in Muller–Hinton agar., (Hi Media, India) by the method introduced by the Clinical and Laboratory Standards Institute (2006) [2]. The 100 µl of test bacterial suspensions which were expected to have the cell density equivalent to 0.5 McFarland standards (approximately 1×108 CFU/ml) were spread uniformly over the surface of the MHA plate. The sterile paper disks (Hi Media, India) with a diameter of 10 mm which were loaded with various concentrations (50–100 µg) of *Vitex trifolia* extracts were placed over the plates and incubated at 37 °C for 24 h and observed for growth inhibition zone [5, 8].

Growth curve analysis

One percentage of overnight bacterial pathogenic cultures (0.4 OD at 600 nm) was inoculated in 250 ml Erlenmeyer flask containing 100 ml of LB broth supplemented with various concentrations (50-100µg) of *Vitex trifolia* extract. The flasks were incubated at the optimum temperature which is suitable for *Pseudomonas aeruginosa* under 180 rpm in a rotatory shaker. The cell density was measured in UV–visible spectrophotometer at every one-hour interval [8].

Biofilm formation in 24-well MTP

The effect of *Vitex trifolia* extract on the biofilm formation on *Pseudomonas aeruginosa* 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 solution (Hi Media, India). The solution was left in MTP wells for 10 min. Then the excess crystal violet solution was removed. So, the crystal violet in stained cells was solubilized with 1 ml of 95% ethanol. The biofilm biomass was quantified by measuring the intensity of crystal violet solution. The intensity was measured at OD 650 nm using UV–visible spectrophotometer.

Biofilm prevention assay

Biofilm prevention assay was done by adding 10 µl of overnight culture of *Pseudomonas aeruginosa* 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 *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 slide, were gently rinsed twice with deionized water (Millipore–Milli-Q) [6].

Protease and elastase assay

Pseudomonas aeruginosa was inoculated in 2 ml LB broth and treated with and without *Vitex trifolia* extract. The culture setup was incubated at 37 °C for 18 h. After incubation, the proteolytic activity of the *Pseudomonas aeruginosa* cell-free supernatant was determined by the azocasein assay [3]. Briefly, 100 ml of either treated and untreated *Pseudomonas aeruginosa* culture supernatant was added to 1ltr 0.3% azocasein (Sigma, St. Louis, MO, USA) in 0.05 M Tris-HC1, 0.5 mM CaCl2 (pH 7.5) and incubated at 37 °C for 15 min. After incubation, ice-cold trichloroacetic acid (10%, 0.5 ml) was added to stop the reaction, followed by centrifugation at 10,000 rpm for 10 min. The absorbance of the clear supernatant was measured at OD 400 nm in a UV-visible spectrophotometer.

The elastolytic activity of the *Pseudomonas aeruginosa* culture supernatant was determined by using the elastase assay [3, 7, 8]. Of the treated and untreated *Pseudomonas aeruginosa* culture supernatant 100 ml was mixed with 900 ml ECR buffer (100 Mm Tris, 1 mM CaCl2, pH 7.5) containing 20 mg Elastin Congo Red (Sigma) and incubated with shaking at 37 °C for 3 h. The reaction was stopped by the addition of 1 ml 0.7 M sodium phosphate buffer (pH 6.0), and the tubes were placed in an ice-water bath. The insoluble ECR was removed by centrifugation, and the absorbance of the clear supernatant was measured at OD 495 nm.

Pyocyanin quantification assay

The *Pseudomonas aeruginosa* culture was inoculated in 5 ml LB broth and cultivated in the presence and absence of *Vitex trifolia* extract at 37 °C for 24 h. After incubation, the culture was centrifuged at 12000 rpm for 2 min and supernatant was extracted. To 5 ml of the supernatant 1 ml of chloroform was added and shaked vigorously. The chloroform layer was taken in a separate tube and 1 ml of 0.2N HCL was added to get pink to deep red solution. Colour absorbance was measured at 520 nm using UV-visible spectrophotometer [3, 4].

EPS quantification

Pseudomonas aeruginosa cells were allowed to form a biofilm on a cover glass (1×1 cm) in the presence or absence of *Vitex trifolia* extract in a 24-well microtiter plate (MTP). The plate was incubated at 37 °C for 18 h. At the end of the incubation, EPS quantification was carried out by total carbohydrate assay. Briefly, glass slides were washed in 0.9% NaCl (0.5 ml) and incubated in an equal volume of 5% phenol (0.5 ml), to which 5 volumes of concentrated H2SO4 were added. The mixture was incubated for 1 h in the dark, and the absorbance was measured at (OD 490) [3].

Swimming assay

The swimming motility of Pseu*domonas aeruginosa* was assessed as described previously [8]. *Pseudomonas aeruginosa* culture was point-inoculated at the center of the medium consisting of 1% tryptone, 0.5% NaCl and 0.3% agar with various concentration of V. trifolia extracts. The plates were incubated at 30 °C in the upright position for a period of 16 h. Swimming motility zones of the bacterial cells were observed.

Light microscopic analysis

One percentage of overnight *Pseudomonas aeruginosa* cultures (0.4 OD at 600 nm) were added into 1 ml of fresh LB medium which containing cover glass of 1 cm2 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% crystal violet 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, Pro Res C5) [2, 3, 8].

RESULTS

Light microscopic analysis

Biofilms are highly dense matrix-encapsulated populations which were attached to the surfaces [9]. The biofilm formation in *Pseudomonas aeruginosa* 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, *Pseudomonas aeruginosa* 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 *Pseudomonas aeruginosa*. The minimum inhibition of biofilm was 50µg/ml and maximum inhibition of biofilm was 100µg/ml. The order, to analyze the anti biofilm efficiency of *Vitex trifolia*,, extract in inhibiting biofilm formation, *Pseudomonas aeruginosa* 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. 2, 3].

Effect on protease, Elastase and pyocyanin production

The attained results with the inhibition of protease with 69% and elastase assay 48% indicated the presence of anti-QS activity of *Vitex trifolia* extract, and therefore the compound was further tested for its ability to reduce QS-dependent pyocyanin production and to analyze the efficiency of the test compound, the *Pseudomonas aeruginosa* cells were cultivated in the presence and absence of *Vitex trifolia* extract. A significant decrease in pyocyanin production of *Pseudomonas aeruginosa* to the level of 85% was observed after treatment with *Vitex trifolia* extract.

Effect on EPS production

Pseudomonas aeruginosa and other bacterial pathogens require EPS as one of their protective barriers in defense to the action of antibiotics, thereby developing resistance to the available antibiotics. Therefore, a reduction in the EPS production would result in an increased susceptibility of *Pseudomonas aeruginosa* cells

to antibiotics. The test compound *Vitex trifolia* extract used in this study caused a 96% decrease in the EPS production of *Pseudomonas aeruginosa*, while maximum EPS production was observed in the control culture.

Effect on swimming motility

The swimming motility of *Pseudomonas aeruginosa* is one of the important requisites for the initial attachment of the cells to the substratum during the biofilm mode of growth [8]. Therefore, a reduction in the swimming motility effectively affects the biofilm forming ability. In the present work, *Vitex trifolia* extract-treated *Pseudomonas aeruginosa* cells exhibited reduced flagellum-driven motility on swimming agar plates, in comparison with the control grown without *Vitex trifolia* extract [fig. 1].

This result indicates that the swimming motility of *Pseudomonas aeruginosa* was inhibited by the action of *Vitex trifolia* extract.

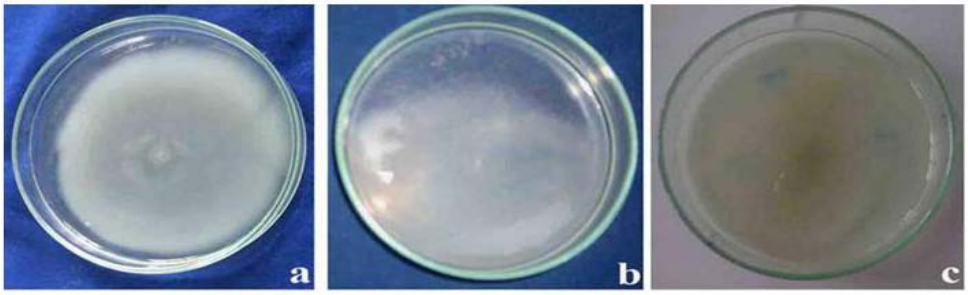

Fig. 1: Swimming motility of *Pseudomons aeruginosa* treated with *Vitex trifolia.* a: untreated control. b: treated with 50 µg of extract. c: Treated with 100µf of extract

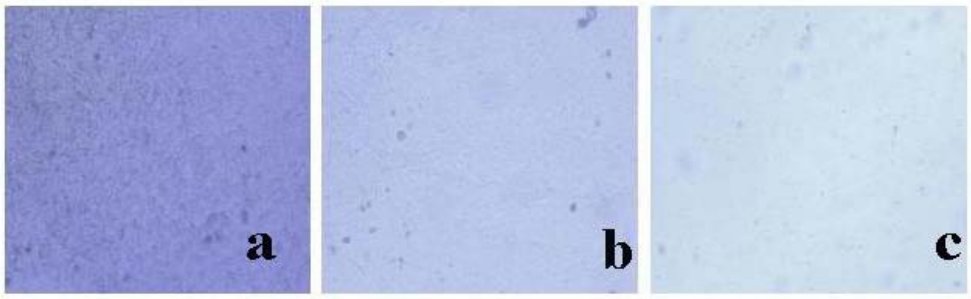

Fig. 2: Light microscopic view of *Pseudomons aeruginosa* treated with *Vitex trifolia*. a: Untreated control. b: Treated with 50 µg of extract. c: Treated with 100µf of extract

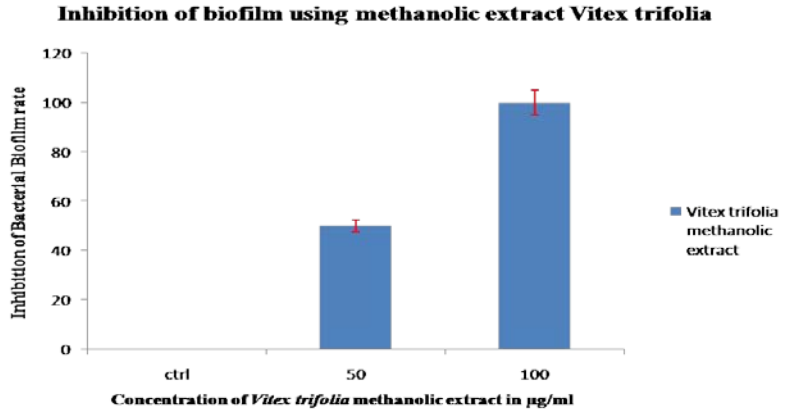

Fig. 3: Graphical representation of biofilm inhibition in *Pseudomonas aeruginosa* using *Vitex trifolia*

DISCUSSION

In the present study, the anti-QS activity of the methanolic extract of *Vitex trifolia* has been shown. As several phenotypic characteristics such as pigment production, motility and biofilm formation in *Pseudomonas aeruginosa* which was regulated by acyl homoserine lactone (AHL)-mediated by QS system. Inhibition of QS by means of anti-QS may in turn, lead to the attenuation of virulence gene expression and subsequent bacterial infections. Although the Quorum sensing activity of *Capparis spinosa* have been investigated so far [7]. Thus, the present investigation has attempted to appraise the efficacy of *Vitex trifolia* to inhibit the QS system. In the continuation of the results attained in the preliminary screening with *Vitex trifolia* extract was further tested for its ability to inhibit biofilm formation, EPS production and flagellar motility in *Pseudomonas aeruginosa*. It has been well reported that AHL-dependent QS plays a major role in the formation of a biofilm with a complex wild type architecture. In the present study, the extract of *Vitex trifolia* inhibited biofilm biomass considerably in a dose-dependent manner [fig. 3] without affecting the bacterial growth. It

has also been proven that surface conditioning promotes surface adhesion and subsequent micro colony formation [4, 11]. Light microscopic images [fig. 2] revealed that the extract of *Vitex trifolia* efficiently reduced the number of microcolonies during the biofilm formation of test bacterial pathogen. Therefore, it is envisaged that treatment of bacterial pathogens with *Vitex trifolia* extracts resulted in the formation of weak biofilms possibly by reducing the surface adhesion and subsequent micro colony formation. Previous studies with the extract of culinary herbs and several plant extracts have been reported to inhibit cell adhesion [11, 8, 7]. There is increasing evidence that AHL mediated QS plays a crucial role in the maturation of biofilms (10) and therefore inhibition of QS can possibly prevent biofilm maturation. Our results are in accordance with previous reports where the secondary metabolites from marine bacteria disturbed the mature biofilms of *Vibrio* spp. [3, 4, 9, 11]. Concerning the EPS, it is a proven fact that EPS plays a major role in the maintenance of biofilm architecture [11]. Moreover, overproduction of EPS leads to alterations in the architecture of biofilm that correlates with an increased resistance of the cells to osmotic and oxidative stresses [4, 9]. Furthermore, there is increasing evidence suggesting that the interference with the expression of EPS synthesis gene leads to the weakening of biofilm architecture [3, 6]. In the present study, the total amount of biofilm EPS was also reduced when bacterial pathogens were treated with extract of *Vitex trifolia* Thus, if the extract of *Vitex trifolia* loosens the architecture of the biofilm by inhibiting the synthesis of EPS, it is possible that resistance of bacterial cells within the biofilm would also be reduced by allowing the penetration of antibiotics. QS-dependent flagellar-driven swimming motility is essential for initiation of cell/surface attachment during biofilm development [1, 4]. The findings of the present study explored the ability of *Vitex trifolia* extract to prevent the swimming migration of test bacterial pathogen in a dose dependent manner [fig. 1]. Flagella could facilitate the swimming motility; inhibition in the flagellar synthesis by the *Vitex trifolia* extract would facilitate the reduced swimming migration pattern. Thus, *Vitex trifolia* indirectly demonstrated consequences on the biofilm formation of the test pathogen in part by interfering with its ability to reach the substratum and subsequent biofilm formation by disturbing AHL-mediated QS system. Results obtained in the present study support the findings of Packiavathy *et al.* [7, 8] in which *Capparis spinosa* inhibited the quorum sensing activity in various pathogens by preventing the initial cell-to-surface attachment. This is the first report on the effect of methanolic extract of *Vitex trifolia* to inhibit the quorum sensing and biofilm formation of bacterial pathogens. Therefore, it is postulated that the biofilm inhibitor could be combined with a conventional antibiotic to efficiently control biofilm by disturbing the mature biofilm and permit the drug to reach the bacterial cells living inside the biofilm.

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CONFLICT OF INTERESTS

Declared None

REFERENCES

1. Annapoorani A, Jabar AKKA, Musthafa SK, Pandian SK, Ravi AV. Inhibition of Quorum sensing mediated virulence factors production in urinary pathogen *Serratia marcescens* PS1 by Marine sponges. Indian J Microbiol 2012;52:160-6.
2. Hejazi A, Falkiner FR. *Serratia marcescens*. J Med Microbiol 1997;46:903-12.
3. Khadar SM, Krishnaswamy B, Shunmugaiah KP, Arumugam VR. 2, 5-Piperazinedione inhibits quorum sensing–dependent factor in *Pseudomonas aeruginosa*. J Basic Microbiol 2012;52:1-8.
4. Kumar RJ, Zi-rong X. Biomedical compounds from marine organisms. Mar Drugs. 2004;2:123-46.
5. Nancy IMR, Meenashree B, Vasanthi J. Screening of antibacterial and qualitative and quantitative analysis of phytochemicals in *vitex trifolia*. Int J Curr Microbiol Appl Sci 2014;3(5):425-31.
6. Nancy IMR, Banu N. Inhibition of Antibiofilm mediated virulence factors production in urinary pathogen *Serratia marcescens* by *Vitex trifolia*. Int J Sci Res 2014;3(8):1972-4.
7. Packiavathy IASV, Agilandeswari P, Musthafa KS. Pandian SK, Ravi AV. Antibiofilm and quorum sensing inhibitory potential of *Cuminum cyminum* and its secondary metabolite methyl eugenol against gram negative bacterial pathogens. Food Res Int 2012;45:85-92.
8. Packiavathy IASV, Agilandeswari P, Ramaswamy RP, Pandian SK, Ravi AV. Antiquorum sensing and antibiofilm potential of *Capparis spinosa*. Arch Med Res 2011;45:85-92.
9. Rice SA, Koh KS, Queck SY, Labbate M, Lam KW, Kjelleberg S. Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. J Bacteriol 2005;187:477-85.
10. Sarah JC, Neil RW, Abigail KPH, David RS, George PCS. Metabolic and regulatory engineering of *Serratia marcescens* mimicking phage-mediated horizontal acquisition of antibiotic biosynthesis and quorum-sensing capacities. Microbiology 2006;152:899-911.
11. Skindersoe M, Epstein EP, Rasmussen TB, Bjarnsholt T, de Rocky Nys, Givskov M. Quorum sensing antagonism from marine organisms. Mar Biotechnol 2008;10:56-3.
12. Thenmozhi R, Nithyanand P, Rathna J, Pandian SK. Antibiofilm activity of coral-associated bacteria against different clinical M serotypes of *Streptococcus pyogenes*. FEMS Immunol Med Microbiol 2009;57:284-94.
13. Traub WH. Antibiotic susceptibility of *Serratia marcescens* and *Serratia liquefaciens*. Chemotherapy 2000;46:315-22.