Advances in life science research

Quercetin from *Cananga odorata* – A Quorum quenching flavonoid inhibiting pyocyanin production in *Pseudomonas aeruginosa*

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ABSTRACT

Quorum sensing is a phenomena of cell density mediated regulation of bacterial gene expression that can control the virulence factors causing pathogenesis.Acyl Homoserine Lactones (AHL) are a group of signaling molecules that regulate quorum sensing in Gram negative bacteria resulting in the control of biofilm formation, pyocyanin production, twitching motility and proteolysis activity.In the present study the pyocyanin pigmentwasproduced from *Pseudomonas aeruginosa*andwas characterized with TLC,FT/IR and LC/MS. Further an attempt was made to extract and purify possible quorum quenching compound from the ethyl acetate extracts ofa highly aromatic and terpenoid containing*Canangaodorata*flower. Theextractaftercharacterization with gradient flash chromatography,TLC, FTIR and LCMS ,was found to contain quercetin, a riboflavin like flavanoid .The effect of this plantextractcontaining quercetin on pyocyanin pigment productionin*Pseudomonasaeruginosa*was evaluated. It could be established that eventhough the plant extract did notcause any significant inhibition on bacterial growth it inhibited the quorum sensing based pyocyanin production in the bacteria. The quorum quenching properties of *Canangaodorata*flower extract carrying quercetin can therefore be considered as a potential therapeutic strategy against *Pseudomonas aeruginosa* mediated infections as this pigment production is a pathogenesis linked property.

KEYWORDS
*Diplostomidmetacercaria;Tetracotylewayanadensis;*WesternGhats;Haludariafasciata;digenetictrematodes

INTRODUCTION

Quorum sensing is a phenomenon that has gained importance in the field of bacterial pathogenesis, colonization, invasion, antibiotic resistance, and emerging diseases.Quorum sensing involves microbial cell to cell communication system within single or mixed microbial population (Chaet al.,1998). Such communication systems are generally established within bacteria, but some fungal species have also shown to perform quorum sensing properties. Apart from this, several plants are also known to produce some metabolites that have the property to mimic the quorum sensing signaling molecules (Gao et al. 2003). These quorum sensing is not only a cell to cell communication between two microorganisms, rather it involves an interacting state between ecological niches comprising of bacteria, fungal and plant species (Teplitskiet al.,2000).
Anti-quorum sensing compounds are also found to be a way of controlling microbial infections by reducing the risk of antibiotic resistance (Leeet al., 2014). Many research works have been performed to isolate compounds from plants possessing medicinal properties that may have anti quorum sensing property. These medicinal plants are rich in novel secondary metabolites that may have anti-microbial and anti-pathogenic activity. The use of these bioactive compounds interferes with the microbes by quenching the QS signaling pathway. These quorum quenching agents could act as a way of bacterial quorum sensing inhibition which can be a potential source in therapeutic application against multi drug resistant bacteria.
Pseudomonas aeruginosa is a gram-negative rod shaped and monoflagellated bacterium that have the ability to survive in a variety of environmental conditions. It is an opportunistic pathogen that can cause a wide range of infections in human beings (Bodeyet al., 1983). These organisms use quorum

sensing systems for producing virulence factors that contribute to its disease-causing ability. It also retains the capability to degrade many pollutants including hydrocarbons and pesticide residues.*Pseudomonas aeruginosa* exhibit quorum sensing signaling pathway which includes three interconnected signaling systems such as las, rhl and pqs. Thesepathways together control the expressions of many virulence factors (Pesci et al., 1999). 3-oxo-C12-homoserine lactone (3-oxo-C12-HSL) in the case of the *las* system, C4-HSL in the case of the *rhl*system, and 2-heptyl-3-hydroxy-4-quinolone in the case of the *pqs*system are the different types of specific signalingmolecules(Pesci et al., 1999). *Pseudomonas aeruginosa* produce phenazine compounds which are redox action pigmentsthatproduce virulence properties. Pyocyanin pigment is one such blue green water-soluble pigment with antibiotic activity produced in active cultures of *Pseudomonas aeruginosa* (Dietrich et al., 2006).

Cananga odorata, commonly known as Cananga tree is a tropical tree found in India and many other areas such as Indonesia, Malaysia and Phillipines. *Canangaodorata*has a variety of medicinal properties and traditional uses. These flowers are used in the treatment of asthma, pneumonia and stomach ache by local communities in certain areas (Hsiehet al. 2012).The phytochemistry of *Canangaodorata*reveals that it contains a variety of flavanoids having natural, complex and volatile compounds which are highly aromatic in nature.Flavanoids are a group of natural products that exhibit a wide range of activities including anti-bacterial, anti-microbial and anti-quorum quenching activities (Bauer and Mathesius, 2004). Many recently reported flavanoid compounds carry anti quorum sensing properties in *Pseudomonas aeruginosa*byspecifically inhibiting certain signaling pathways (Pesci et al.,1999; Hisham et al.,2020). In the present study an attempt was made to explore the possibility of finding out a quorum quenching flavonoid from *Canangaodorata* flower for regulating pyocyanin synthesis in *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Strain

The strain used in this study was *Pseudomonas aeruginosa* obtained from the culture collections of Bioprocess and Biotechnology lab, School of Biosciences,Mahatma Gandhi university, Kottayam.

Pyocyanin production in *Psedomonas aeruginosa*

Pseudomonas aeruginosa was inoculated in Kings B broth(Pigment production medium)at pH of 7.5 and incubated for 18 to 24 hours to get the blue green coloured pigment. The supernatant obtainedafter centrifuging at 10000 RPM for 15 minuteswassubjected to solvent extraction with chloroform and acidified with 0.1 N HCl((Pesci et al., 1999).). 2 ml of extracted pyocyanin pigment was taken for measuring optical density at 520 nm. The absorbance was then multiplied with the extinction coefficient and the yield of pyocyanin was calculated.

Analysis of extracted pyocyanin

The extracted pigment was further confirmed by TLC with methanol and chloroform (1:1) as mobile phase and the Rf value of the pigment was calculated. The pigment extract was subjected to FT/IR (Shimadzu, IR Prestige-21 Spectrometer) between 4000 cm^{-1} and 400 cm^{-1} for determination of functional groups and was also subjected to LC-MS analysis. The sample was analysed using Acquity H class UPLC with BEH C18 column (50 mm×2.1mm×1.7 µm) and Xevo G2 (Waters) Quadruple Time of Flight (Q-TOF) with methanol and water (0.3 ml mil^{-}) as mobile phase. All the spectra were recorded in both positive and negative ionization modes between mass ranges of 50-600 Da.

IsolationofAHL mimics from *Cananga odorata* flower

10 g *Cananga odorata*flower was surface sterilized with 5% active chlorine hypochlorite solution, washed thoroughly with distilled water and dried under aseptic conditions. These dried flowers were made in to fine power with the help of mortar and pestle. The extraction of flower was done in Methanol, Ethyl acetate, Water, Acetonitrile and diethyl ether.The flower extracts in different solvents wereanalyzedbysilicaplated thin layer chromatography with 10 µl sample having methanol and water (6:4) as mobile phase. Riboflavin was taken as the standard.

Characterization of AHL mimic compound from *Cananga odorata* flower

The ethyl acetate extract was again purified using Gradient Flash Chromatography.The sample adsorbed on the silica was eluted stepwise at a flow rate of I $ml^{-1}$with 50 ml of hexane followed by linear gradient of 50 ml of hexane:ethyl acetate (40:10 to .0:50 v/v),50 ml of ethyl acetate:methanol (40:10 to 0:50 v/v) ,50 ml of methanol:acetonitrile (40:10 to 0:50 v/v) and 50 ml of acetonitrile:water (40:10 to 0:50 v/v).The separated fractions of flash chromatography wereestimated for absorption maximum (λ_{max}) using UV-Vis Spectrophotometer (UV-2600,Shimadzu, Japan) between 700nm and 200 nm. The maximum wavelength of riboflavin standard was taken as the control. Hexane ethyl acetate fractionwasselectedon comparing with the riboflavin standard and was subjected to FT/IR and LC/MS analysis as mentioned earlier.

Effect of AHL mimic flavonoid compound from *Cananga odorata* on pyocyanin production in *Pseudomonas aeruginosa*

The hexane ethyl acetate fraction at different concentrations (0.5µl, 1.0 µl and 1.5µl) were supplemented in to KingsBmediuminoculated with the strain and incubated for 18 to 24 hours for evaluation of pigment production. After incubation the broth was centrifuged for 10000 RPM for 15 minutes and supernatant was taken for measuring OD at 520 nm for detecting the presence of pyocyanin.

RESULTS

Evaluation of pigment production in *Pseudomonas aeruginosa*

Kings B broth at pH of 7.5 was inoculated *Pseudomonas aeruginosa* and incubated 18 to 24 hours to get a blue green colouredpyocyaninpigment. The absorbance at 520 nmas then multiplied with the extinction coefficient 17.072 and the yield of pyocyanin was calculated as 2.082784 µg/ml. The extract was submitted to TLC analysis (Fig.1) and the single spot on FT/IR (Fig.2) analysis indicated the presence of pyocyanin which was further confirmed with LC/MS.

Figure 1.TLCanalysis of the chloroform extract of the supernatant of the Kings B medium inoculated and incubated with pyocyanin producing *Pseudomonas*

aeruginosa.

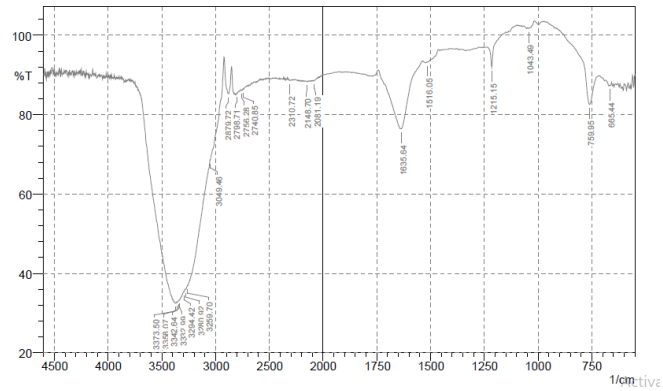

Figure 2.FT/IR analysis of the chloroform extract of the supernatant of the Kings B medium inoculated and incubated with pyocyanin producing *Pseudomonas aeruginosa.*

The FTIR spectra of the extracted pigment showed bands between 3400 cm^{-1} and 3250 cm^{-1} in spectra and this was an indicative of OH groups, bands between 2700 $cm^{-1}$was indicative of C-H stretch of the aromatic compounds.The band at 1516 cm^{-1}represented C=N and a band at 1215 $cm^{-1}$was indicative a C-N group. These representations confirmed the presence of phenazine ring characteristic of pyocyanin.

The LCMS data confirmed that the blue colour pigment present was pyocyanin.The peak at retention time 6.346 showed a fragmentation at m/z 214.2304 (M+H+) characteristic of pyocyanin.

Isolation of quorum quenching compounds from *Cananga odorata* flower

Plants contain a variety of phytoconstituents. Some of the plant flavonoids including riboflavin are proved to be a quorum sensing regulator in *Pseudomonas aeruginosa*. Flower samples extracted with different solvents were subjected to thin layer chromatography taking riboflavin as standard to check the presence of flavonoid like compounds that may have AHL regulating activity.Out of this, ethyl acetate extract showing aprominent band similar to riboflavin was selected for further studies.The ethyl acetate fraction was purified using silica gel column chromatography and was eluted with different solvents. The fractions collected after flash chromatography were analyzed by UV-VIS Spectroscopy and the hexane ethyl acetate fraction with absorption maxima similar to riboflavin was selected for further studies

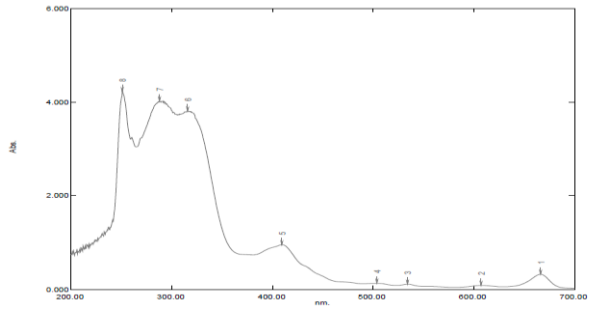

Figure 3.UV Vis spectrum of hexane ethyl acetate fraction (40:10) of gradient flash chromatography performed with the chloroform extract of *Cananga odorata* flower

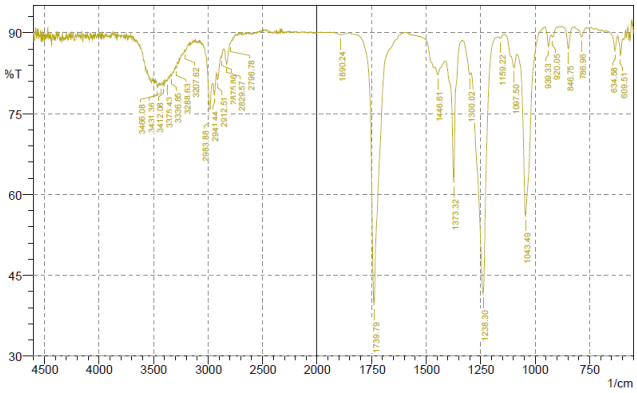

Figure 4.FT/IR analysis of hexane ethyl acetate fraction (40:10) of gradient flash chromatography performed with the chloroform extract of *Cananga odorata* flower

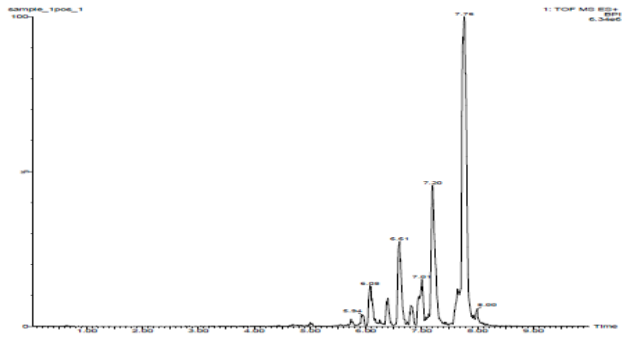

Figure 5.LC of hexane ethyl acetate fraction (40:10) of gradient flash chromatography performed with the chloroform extract of *Cananga odorata* flower

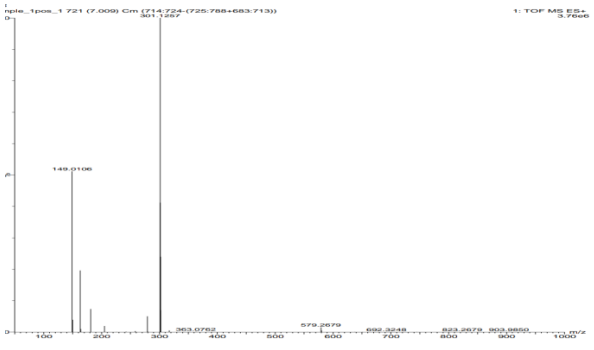

Figure 6.LC/MS analysis of the peak at 7.01 min in the LC of hexane ethyl acetate fraction obtained from gradient flash chromatography performed with the chloroform extract of *Cananga odorata* flower

The LCMS of the extract showed seven prominant peaks between retention times 5.94 min and 8.0 min in chromatogram. The mass spectra at retention time 7.009 min peak at m/z 301.257(M+H+) was closed to the mass (302.236) of a quorum quenching flavanoid compound Quercetin.

Effect of quorum sensing regulation of pyocyanin production of these two fractions

Hexane ethyl acetate fraction obtained from the gradient flash chromatography of the extract of *Cananga odorata* flower was added at different concentrations to the pyocyanin production medium inoculated with*Pseudomonas aeruginosa*. Upon incubation none of the medium could show the presence of the pigment but carried only the bacterial growth.

DISCUSSION

Bacterial quorum sensing involves cell to cell communication and gene expression within a bacterial population. The virulence factors in bacteria such as biofilm formation, pyocyanin production and protease production are Quorum Sensing regulated mechanism in Gram negative bacteria which carry Acyl Homoserine Lactones (AHLs)as signaling molecules (Chaet al., 1998).*Pseudomonas aeruginosa* is a gram-negative quorum sensing bacterium and the quorum sensing mechanisms are dependent on luXI/luR genes (Steidleet al., 2002)

Quorum Quenching is also becoming awide area of research and this could also be exploited for the regulation of AHL mediated signaling molecules involved in the control of virulence factors. The quorum quenching molecules that act in gram negative bacterial species like *Pseudommonas*sp. have structural similarity with AHL molecules and therefore regarded as AHL mimic compounds(Paluchet al.,2020).Flavanoid compounds are found to have quorum sensing inhibition in many bacterial strains. Nine flavanoid compounds were isolated from *Piper delineatu*and*Renealmiaalpinia* which inhibited the violaecin production in QS regulated *Chromobacteriumviolaecium.*Quercetin.a naturally occurring flavanoid compound in fruits and vegetables wasfound to have antitoxic and anticarcinogenic properties. Quercetin was found to reduce the ability of elastase and biofilm production in *Pseudomonas aeruginosa* (Ouyanget al.,2016).

The present study focused on the possibility of quorum quenching in regulating the productionof pyocyanin, a virulence factor in *Pseudomonas aeruginosa* using the extract of *Canangaodorata*flower.The phytochemicals present in the extracts of *Canangaodorata*flowercould have quorum quenching activities as it carried many flavonoid compounds.

Pseudomonas aeruginosa was evaluated for its QS mediated pyocyanin production King's B medium. The strain could produce the blue green pigment at pH 7.5.The pigment was extracted and quantified with TLC and FTIR. The LCMS analysis of the pigment gave a molecular mass of *m/z*214 $(M+H^{+})$ representing pyocyanin.

*Canangaodorata*flowers were extracted with different solvents and TLC was done taking riboflavin as standard. Ethyl acetate extract was then purified using gradient flash chromatography and several fractions were obtained in different solvent ratios.UV- Vis spectrum of the collected fractions was taken and compared with the UV spectrum of riboflavin (Rajamaniet al.,2008). Riboflavin have been identified as a AHL mimic flavonoid compound in many of the quorum sensing related activities of *Pseudomonas aeruginosa* and hence in this study riboflavin has been taken as a standard reference for screening out structurally similar compound that can act as quorum sensing regulators. Hence out of the various fractions obtained,thefraction having a similarity with the UV spectrum of riboflavinwas selected for further studies.

Further TLC, FTIR and LCMS analysis confirmed the presence of the flavanoidcompound,quercetin with a molecular mass of 301.256 in the extract. Subsequently the effect of the quercetin fraction on pigment production in thebacterialstrain was analyzed. By keeping the same conditions,the pigment production media was supplemented with various concentrations of the flower extracts of*Canangaodorata*along with the inoculum of *Pseudomonas aeruginosa*. The results obtained showed that the quercetin containing plant extract could inhibit pyocyanin production without affecting the bacterial growth. Quercetin, $C_{15}H_{10}O_7$ with a molecular mass of 302 could have interacted with the signal receptor for the specific AHL (3 oxo C 12 HSL with molecular weight 297) involved in the regulation of the synthesis of pyocyanin pigment. This could be resulted in the inhibition of Quorum sensing and subsequently the inhibition of the synthesis of the pigment.

An approach of using plant derived natural components to control bacterial activitiesthrough quorum quenching could be a promising strategy for controllingbacterial infections. The present finding thatquercetin from the flowers of *Cananga odorata*could be used for inhibiting pyocyanin production in *Pseudomonas aeruginosa*throws light into the possibility of formulating new molecular methods for manipulating bacterial activity with plant derived metabolites.

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Conflict of interest

The authors declare that we have no conflict of interest in the research findings submitted.

Human and Animal Rights

This article does not contain any study with human participants or animals in the experiments performed by any of the authors

REFERENCES

BauerWD, Mathesius U (2004) Plant responses to bacterial quorum sensing signals. Curr. Opin. Plant Biol., 7, 429-433.

Bodey GP, Bolivar R, FainsteinV,Jadeja L (1983) Infections caused by *Pseudomonas aeruginosa*. Rev Infect Dis.,5(2),279-313.

Cha C, GaoP, Chen YC, Shaw PD, Farrand SK (1998) Production of acyl-homoserine lactone quorum sensing signals by gram-negative plant associated bacteria. Mol. Plant Microbe Interact., 11,1119-1129.

Dietrich, Lars EP, Alexa Price-Whelan, Ashley P, Marvin W, Newman DK (2006) The phenazine pyocyanin is a terminal signaling factor in Quorum sensing network of*Pseaudomonas aeruginosa.* Mol. Microbiol.,61(5), 1308-1321.

Gao M, TepliskiM, RobinsonJB, Bauer WD (2003) Production of substances by *Medicago trunculata* that affect bacterial quorum sensing. Mol. Plant Microbe Interact., 16, 827-834.

Hisham AA,Moutus AS, Dalla E (2020) Curtailing quorum sensing in *Pseaudomonas aeruginosa* by sitagliptin. Curr. Microbiol.,77,1051-1060.

Hsieh TJ, Chang FR, Chia YC, Chen CY, Chiu HF, W YC (2012) Cytotoxic constituents of the fruits of *Canangaodorata*. J. Nat. Prod., 64(5) 616–619.

Lee K, Lee JH, Kim SI, Cho MH, Lee J (2014) Antibiofilm, anti-hemolysis, and anti-virulence activities of black pepper, Cananga, myrrh oils, and nerolidol against *Staphylococcusaureus*.Appl. Microbiol. Biotechnol., 98(22), 9447–9457.

Ouyang J, Sun F, Feng W,Sun Y, Qiu X, Xiong L, Liu Y, Chen Y (2016) Quercetin is an effective inhibitor of quorum sensing,

biofilm formation and virulence factors in *Pseaudomonas aeruginosa.* J.Appl. Microbiol., 120(4),966-974.

PaluchEJ,Rewak-SoroczynskaI, JedrusikE, MazurkiewiczK, Jermakow (2020) Prevention biofilm formation by quorum quenching. Appl. Microbiol. and Biotechnol., 104,1871-1881.

Pesci EC, MilbankJBJ, Pearson JP, Mc Knight S, KendeAS, Greenberg EP, Iglewski BH (1999) Quinolone signaling in the cell- to-cell communication system of *Pseudomonas aeruginosa.* Proc. Natl. Acad. Sci.,96, 11229-11234.

Rajamani S, Bauer WD, Robinson JB, Farrow JM,Pesci EC, TeplitskiM, Gao M, Sayre RT, PhillipsDA (2008) The vitamin riboflavin and its derivative lumichrome activate the LasR bacterial quorum –sensing receptor. Mol. Plant Microbe Interact.,21(9),1184-92.

SteidleA, Allesen-HolmM, RiedelK,BergG,SorenMG (2002) Identification of an N-acylhomoserine lactone – dependent quorum –sensing system in *Pseudomonas putida* strain IsoF. Appl. Environ. Microbiol., 68(12), 6371- 6382.

Teplitski M, Robinson JB, Bauer WD (2000) Plants secrete substances that mimicbacterial*N*-acyl homoserine lactone signal activities and affect population density-dependent behaviors in associated bacteria. Mol. Plant Microbe Interact, 13, 637-648.