



# **Attenuation of Quorum Sensing Controlled Virulence Factors and Biofilm Formation by Edible Fruit Extract of *Coccinia indica* against *Pseudomonas aeruginosa***

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## **Authors' contributions**

This work was carried out in collaboration among all authors. Author PSG did the study design. PSG and SD did the preparation of extract and determination of biofilm inhibition assay and quantification of pyocyanin production in *Pseudomonas aeruginosa*. The Data collection was done by author PSG and SD. Author SD wrote the manuscript and it was edited and revised by author PSG, SD, ASSG and JVP approved submission of Manuscript. All authors read and approved the final manuscript.

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## **ABSTRACT**

**Background:** *Pseudomonas aeruginosa* is a Gram-negative opportunistic human pathogen that mainly infects immunocompromised individuals and patients with urinary tract infection and chronic infections of the respiratory pathways, including cystic fibrosis. Many quorum sensing (QS) controlled components such as bio surfactants and swarming motilities play an important role in the establishment of biofilms. Targeting these factors through anti-QS strategies prevent biofilm formation and treating infections. *Coccinia indica* commonly called little gourd is used to treat diabetes, wound, burn infections and has antioxidant, antibacterial and antitussive properties.

**Methods:** The methanolic fruit extract of *C. indica* was prepared and screened for anti-QS and

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anti-biofilm formation activity. Pyocyanin inhibition, rhamnolipid, crystal violet staining assay tests was performed and the extract was observed under fluorescent microscope.

**Results:** The results obtained are as follows - the fruit extract inhibits the pyocyanin at 58.13% and 42.27% at 0.5 mg/ml and 1.0 mg/ml, biofilm at 69.86% and 49.06% at 0.5 mg/ml and 1.0 mg/ml, inhibits rhamnolipid assay and under fluorescent microscope it is seen scattered whereas control produce biofilm matrix like appearance.

**Conclusion:** Since less study has been made on the quorum sensing and biofilm activity of *C.indica* our study aimed to fulfil it and it was found that it exhibits good biofilm formation and thus can be used for treating infections.

**Keywords:** *Biofilm formation; Coccinia indica; Pseudomonas aeruginosa; Quorum sensing; Environment.*

## 1. INTRODUCTION

Bacteria usually monitor the environment in which they live constantly for changes. These attain their own critical species population density by cell to cell chemical signaling thereby controlling the expression of specific genes. This process of gene regulation can be called quorum sensing. Many Gram-negative bacteria use N-acyl homoserine lactones (AHLs) as the signaling molecules synthesized by LuxI-type synthase. They differ from one another in terms of length and substitution on their acyl side chains [1]. *Pseudomonas aeruginosa*, type of gram negative opportunistic bacteria causes majority of the secondary infections in immunocompromised patients having cystic fibrosis, HIV, burn wound producing maximum mortality rate and is responsible for about 57% of the total nosocomial infections by producing both extracellular virulence and cell-associated factors through these well defined quorum sensing systems with *las* system that uses *N*-(3-oxododecanoyl)-L-homoserine lactone as the signal molecule and *rhl* system that functions by *N*-butanoyl-L-homoserine lactone ( $C_4$ -HSL) as the signal molecules. Intermediate present between them is the quinolone system that uses 2-heptyl-3-hydroxy-4-quinolone as the signal molecule [2]. These molecules control and release various virulence factors (such as pyocyanin, Las A, Las B, rhamnolipid, exopolysaccharide, colony morphology etc.), toxins, bioluminescence, antibiotic resistance and biofilm formation.

It is a highly adaptable organism colonizing a wide niche variety and it is one of the main pathogen in CF patients and the infections caused by it can be widely classified into acute and chronic infection. Acute infections characterized by motility, cytotoxicity and rapid involvement of disease whereas chronic

infections closely characterized by slower growth, biofilm formation and antibiotic resistance [3]. Its capacity to form biofilm is considered an important requirement for the formation of chronic colonization in human tissues mainly mediated by type 4 pili and flagella. Three genes of the bacteria are identified which are collectively called cups. These are cup A, cup B based on their chaperone usher fimbrial assembly. But only cupA gene cluster is required for the formation of biofilm [4].

Bacterial communities proliferate by forming biofilms which are closely linked to quorum sensing [5]. QS has been considered as an important target for improving unique anti infective means that does not depend on the use of antimicrobials [6]. This biofilm forming ability of *P. aeruginosa* makes the infection persist. *P. aeruginosa* that lacks in producing *las* signal molecules produces biofilm that is more thinner and does not display the three dimensional structure as detected in the parent [7]. The biofilm formed by *P. aeruginosa* under flow through conditions produced heterogeneous with mushroom-shaped micro colonies where glucose was used as the carbon source and flat and uniform when citrate is used as a carbon source [8]. Biofilms are complex microbial cells that are embedded in the extracellular matrix and composed of proteins, DNA, extracellular polysaccharides etc thereby providing a protective lifestyle. The components of biofilm in *P. aeruginosa* are alginate, *Psl* and *Pel*. Alginate is required for polymerization and *Psl* provides cell to cell surface interaction in the initiation of biofilm formation and protection of its structure [9]. It protects the bacterial cell through host defense mechanism and antimicrobial therapy [10].

*Coccinia indica* belonging to the cucurbitaceae family is commonly known as little gourd and is

used extensively in the Indian Ayurveda and Unani. They are found to be long, fleshy with tuberous roots and smooth green fruits[11]. Leaves are mainly used to treat diabetes, ulcer, wound, asthma and have antioxidant, antibacterial and antitussive properties etc [12]. The phytochemical constituents present in the fruit are alkaloids, steroids, tannins, glycosides, phenol, terpenoids, flavonoids etc. Every part of the plant is found to be valuable in the field of medicine for treating psoriasis, ringworm, scabies, smallpox etc and hence considered as a wild indigenous vegetable [13]. The phytochemical constituent comprises resins, flavonoids, proteins and fatty acids as the main components. Aspartic acid, Glutamic acid, Histidine, arginine and valine has also been found along with the phytochemical constituents[14]. The fruits are considered to be fusiform-ellipsoid shape, slightly beaked and marked with white streaks when immature and a bright scarlet when fully ripe[15]. Our team has extensive knowledge and research experience that has translated into high quality publications [16–27],[28–32].[33][34][35].[36–40] Hence, the study aims in finding the quorum sensing and the biofilm forming ability of fruit extract against *P. aeruginosa*.

## 2. MATERIALS AND METHODS

The study was conducted at Saveetha Dental College under the supervision of a guide and laboratory assistant. Ethical approval was not needed to conduct the research study.

### 2.1 Bacterial Stain and Culture

Wild type of *P. aeruginosa* isolate was obtained from the patients samples and grown in a sterile Luria Bertani (LB) broth at 37°C and then kept in a rotary shaker (120 rpm) for 24 hrs.

Plant product (Fruit) and preparation of the fruit extract

Fruits of *C. indica* were collected from a local market nearby in Chennai, Tamilnadu, India. They were sealed in a sterile polythene bag and brought to the laboratory where they were air dried, shaded, blended and finally made into powder form.

### 2.2 Methanol Extraction

100ml of the methanol was mixed with 10g of the powder prepared and kept in incubation for about

48 hrs at normal room temperature 37°C on a rotary shaker of 120 rpm. After that, the filtrate was extracted using Whatman No. 1 filter paper and dried further at 50°C on a rotary flash evaporator to remove methanol and a crude extract of the fruit is obtained which is then stored at 4°C.

### 2.3 Evaluation of MIC (minimum inhibitory concentration) of Crude Extract

MIC of the crude methanol extract of *C. indica* was evaluated by using a serial two fold dilution method. They were assessed under different concentrations ranging from 0.25mg/ml,0.5mg/ml,1mg/ml to 2 mg/ml. The growth of the bacteria was then found by adding 2,3,5-triphenyltetrazolium chloride that acts as an indicator. The lowest concentration where no visible growth is observed is considered to be the MIC.

### 2.4 Quantification of Pyocyanin

The quantitative analysis of the methanolic fruit extract on quorum sensing controlled pyocyanin production in *P. aeruginosa* was determined using spectrophotometry. An 18hr culture of bacteria was also kept in LB broth with both in the presence and absence of the fruit extract and left for incubation at 37°C for 24h. Later, it was dissolved in 200µl of DMSO. The cell debris was removed by centrifugation and the absorbance of pyocyanin was measured at 585nm using microtitre ELISA plate reader. The percentage of pyocyanin inhibition was then calculated and compared with a standard control by measuring it at 600nm after treating with sub MIC of antimicrobial extract. The formula to be calculated is as follows

$$\% \text{ pyocyanin inhibition} = \frac{\text{assay control OD at 585} - \text{unknown sample OD at 585 nm}}{\text{Control OD at 600}} \times 100$$

### 2.5 Crystal Violet Staining of Biofilm Assay

Inhibitory effect of *C. indica* on biofilm formation on *P. aeruginosa* was analyzed by using a static microtitre plate. It was grown in TSB for 18hrs at 37°C on a rotary shaker at 150rpm. 20µl cultures were then transferred to M9 minimal media with 0.4% of glucose along with autoinducers and the bacterial culture were dispensed into wells and further incubated at 37°C without agitation to

detect the biofilm formation by staining with 100µl crystal violet and incubated for 15 min at normal room temperature 37°C and the absorbance was measured using spectrophotometer.

## 2.6 Rhamnolipid Assay

*P. aeruginosa* was treated with *C. indica* at sub MIC 0.3% (v/v). After incubation, 20µl treated and non - treated cultures were added to the wells containing 1mM of MgSO<sub>4</sub>, glucose, CTAB etc. The plate was then observed in the presence of a clearance zone to find the precipitate around the dark blue colonies, indicating rhamnolipid production by the fruit extract of *C. indica*.

## 2.7 Fluorescence Microscope

*P. aeruginosa* is grown in TSB for 18hrs at 37°C on a rotary shaker. Then 20µl is transferred to M9 minimal media with 0.4%(w/v) of glucose. A 18mm glass coverslip was kept over 50nm sterilised petri plate and TSB is vigorously mixed with methanol extract and poured into the petri dish with the cover plate. A control plate without the extract was also included and they were incubated for 24h at room temperature. The cover slip is then rinsed using distilled water, air dried, then stained with 0.1% of acridine orange for 2.5 min. The test and control slip is then viewed under a fluorescent microscope for biofilm formation. Permission

## 3. RESULTS AND DISCUSSION

The synthesis of *P. aeruginosa* is regulated by the QS system. Fig 1 showed the inhibition of pyocyanin pigment by *C. indica* and Fig 2 shows that pyocyanin is inhibited by 58.18% at 0.5mg/ml and 42.27% at 1mg/ml. Hence, the end point or MIC of *C. indica* was found to be 0.5mg/ml. Though inhibition takes place even after the end point the percentage of inhibition is found to be decreased. Previous study has quoted that the methanolic extract of *A. pungens*, *C. colocynthis* exhibited better MIC, hence pyocyanin was determined with sub MIC of methanolic fruit extract[41]. Similarly, our study also showed that sub MIC of methanolic extract of *C. indica* provided better percentage of inhibition.

### 3.1 Effect of QS Mediated Virulence Factors in *P. aeruginosa* by fruit Extract

It was seen that the methanolic extract of *C. indica* inhibited growth at 0.5mg/ml whereas a study has reported that *C. violaceum* inhibited growth at 5mg/ml[42]. But in our study the extract was found to show promising activity of anti QS at sub MIC level in dose dependent manner as concentration increases at 58.13% and 42.27% in presence of 0.5mg/ml when compared to control.

### 3.2 Effect on Biofilm Formation

Fig3 shows the percentage of inhibition of biofilm formation at 69.86% and 49.06% in presence of 0.5mg/ml and 1mg/ml respectively showing significant reduction in biofilm growth as concentration increases in dose dependent manner. A similar study was found where the test concentrations (MIC, 2x MIC, 4 x MIC) of methanolic fruit extract showed a reduction in the formation of biofilm by oral strain of *S. aureus* on polystyrene in a remarkable manner[43].

### 3.3 Effect on Rhamnolipid Assay

Fig 4 shows rhamnolipid assay of the fruit extract. It is seen that the control produced large zone around the dark blue colonies whereas the methanolic fruit extract of *C. indica* showed less amount of ppt around the colony indicating that the fruit inhibits rhamnolipid. It is considered that rhamnolipid is important for formation and maintenance of biofilm in *P. aeruginosa* and is under QS system control. Treating with anti QS results in decreased production by type bacteria. *M. koenigii* also effectively reduces the rhamnolipid production in contrast to the control[44].

### 3.4 Fluorescent Microscope

Fig 5 shows the appearance of the fruit extract under fluorescent microscope. It was seen that the control produced a green matrix-like appearance while the treated fruit extract showed no matrix formation at 0.5mg/ml and was found scattered all over. The present study was done mainly to determine the in vitro antibacterial activity of natural plant extract so as to evaluate the scientific basis of the applications. Ethanolic extract of similar fruit of *C. indica* called *C. grandis* that belong to the same family showed high antibacterial activity against *S. aureus*, *E.*

*coli*, *K. pneumoniae* and *S. pyogenes*[45]. But in our study, the methanolic extract of *C. indica* was found to produce good antibacterial activity and biofilm formation against *P. aeruginosa*. Hence, it can be used to treat diseases concerning bacterial infections. It was also found that the crude extract of *C. indica* produced moderate antibacterial activity against all the gram positive and negative bacteria mainly with *S. aureus* tested with the zone of inhibition ranging from 1 - 19mm[46]. Previous studies have also shown the significant activity of methanolic and ethanolic extract against various bacteria and fungi and found that methanol was a better solvent for

extraction and crude extract preparation and isolation of phytochemicals having antimicrobial and antibiofilm activity[47] (43). Since less study has been made on the quorum sensing activity and biofilm formation, our study was done mainly to create awareness and knowledge about the biofilm formation and anti QS sensing activity of *C. indica* on *P. aeruginosa*. The limitation of the study was small sampling size, study was conducted *in-vitro* and not *in-vivo* and it was tested against only one type of bacteria. So all these limitations can be overcome by further developing the present study for its effectiveness.

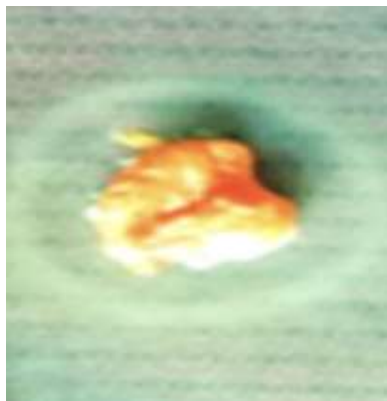


Fig. 1. Preliminary screening of *Coccinia indica* fruit inhibited pyocyanin pigment production in *P. aeruginosa* PAO1

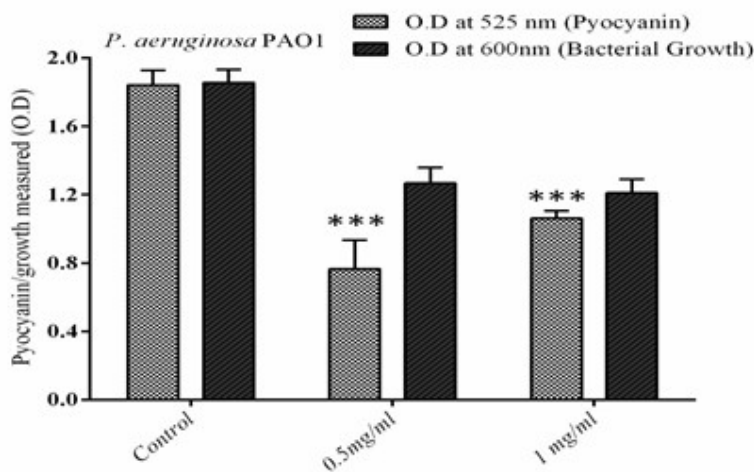


Fig. 2. Pyocyanin inhibition assay: At different concentration of *C. india* extract inhibited the pyocyanin pigment

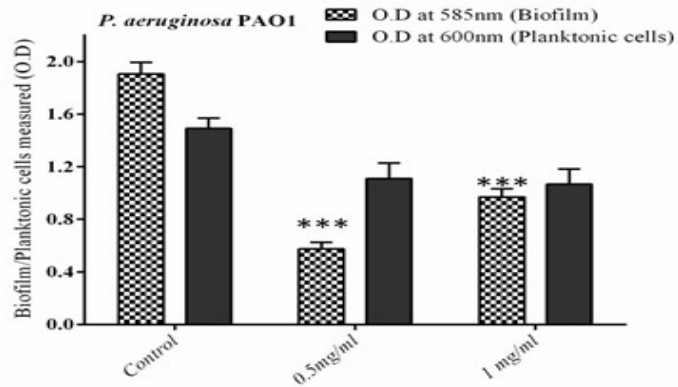


Fig. 3. Biofilm inhibition assay: At 0.5mg/ml - 69.86%; 1mg/ml- 49.06% inhibition

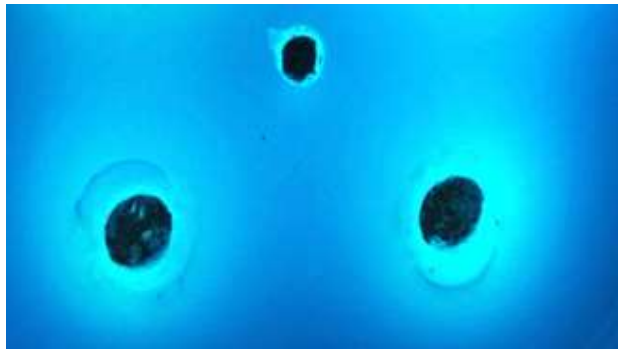


Fig. 4. Rhamnolipid assay: Less precipitate of rhamnolipid was observed around the well (treated at 0.5mg/ml) and more precipitate of rhamnolipid was observed around control well

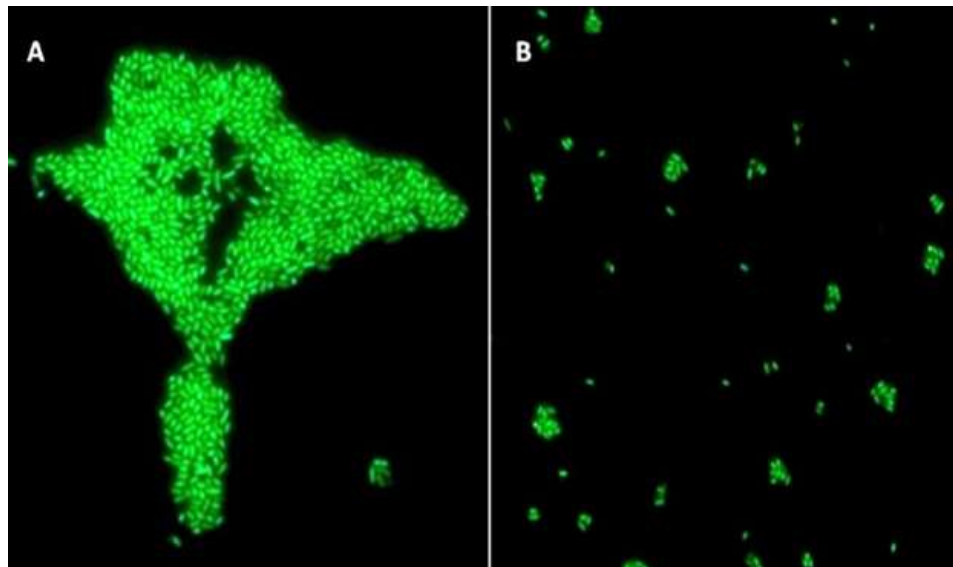


Fig. 5. Fluorescence microscope image : a) Control b) Treated (0.5mg/ml)

#### 4. CONCLUSION

Hence, the present concluded that the methanolic fruit extract of *C. indica* has good anti-QS and antibiofilm activity against *P. aeruginosa* and it can prevent biofilm formation and bacterial pathogenesis. Further in vivo and clinical trials need to be made for standard drug development to combat diseases.

#### DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

#### CONSENT

It is not applicable.

#### ETHICAL APPROVAL

It is not applicable.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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