Guava Leaf Extract Inhibits Quorum-Sensing and Chromobacterium violaceum Induced Lysis of Human Hepatoma Cells: Whole Transcriptome Analysis Reveals Differential Gene Expression

Runu Ghosh, Bipransh Kumar Tiwary, Anoop Kumar, Ranadhir Chakraborty
OMICS Laboratory, Department of Biotechnology, University of North Bengal, Siliguri, West Bengal, India

Abstract
Quorum sensing (QS) is a process mediated via small molecules termed autoinducers (AI) that allow bacteria to respond and adjust according to the cell population density by altering the expression of multitudinous genes. Since QS governs numerous bioprocesses in bacteria, including virulence, its inhibition promises to be an ideal target for the development of novel therapeutics. We found that the aqueous leaf extract of Psidium guajava (GLE) exhibited anti-QS properties as evidenced by inhibition of violacein production in Chromobacterium violaceum and swarming motility of Pseudomonas aeruginosa. The gram-negative bacterium, C. violaceum is a rare pathogen with high mortality rate. In this study, perhaps for the first time, we identified the target genes of GLE in C. violaceum MTCC 2656 by whole transcriptome analysis on Ion Torrent. Our data revealed that GLE significantly down-regulated 816 genes at least three fold, with p value p<0.01, which comprises 19% of the C. violaceum MTCC 2656 genome. These genes were distributed throughout the genome and were associated with virulence, motility and other cellular processes, many of which have been described as quorum regulated in C. violaceum and other gram negative bacteria. Interestingly, GLE did not affect the growth of the bacteria. However, consistent with the gene expression pattern, GLE treated C. violaceum cells were restrained from causing lysis of human hepatoma cell line, HepG2, indicating a positive relationship between the QS-regulated genes and pathogenicity. Overall, our study proposes GLE as a QS inhibitor (QSI) with the ability to attenuate virulence without affecting growth. To the best of our knowledge, this is the first report which provides with a plausible set of candidate genes regulated by the QS system in the neglected pathogen C. violaceum.

Citation: Ghosh R, Tiwary BK, Kumar A, Chakraborty R (2014) Guava Leaf Extract Inhibits Quorum-Sensing and Chromobacterium violaceum Induced Lysis of Human Hepatoma Cells: Whole Transcriptome Analysis Reveals Differential Gene Expression. PLoS ONE 9(9): e107703. doi:10.1371/journal.pone.0107703

Introduction
With the increase in the number of multi-drug-resistant pathogenic bacteria worldwide, there is a dire need for developing strategies to fight bacterial infections. The indiscriminate use of novel antibiotics that interfere with the metabolism of bacteria have only added to this number. Since Quorum Sensing (QS) regulates many virulence determinants of various pathogens, it has emerged as an attractive target to control their pathogenicity [1,2,3]. QS is a cell-to-cell communication mechanism, regulated by small diffusible signalling molecules termed autoinducers (AI), which allows bacteria to respond and adjust their needs in a population density-dependent manner by altering the expression of multitudinous genes [4,5,6]. The AIs used by Gram-negative bacteria are known as N-acyl homoserine lactones (AHLs), while Gram-positive bacteria utilize post-translationally modified oligo-peptides as signaling molecules [7,8]. In most Gram-negative bacteria, QS systems are based on LuxI/LuxR homologues. The LuxI homologues encode an AHL synthetase involved in the synthesis of signal molecules, and the LuxR homologues encode the transcriptional regulatory protein which, upon binding of the cognate signal molecules, activates the transcription of the QS target genes [9].

Chromobacterium violaceum, an opportunistic pathogen, is a free-living, gram-negative, facultative anaerobic β-proteobacterium commonly found in water and soil in the tropical and subtropical regions [10]. In human, C. violaceum infection is rare, but this may be attributed to under-reporting of such cases in areas where the risks of exposure are high and diagnostic facilities are scarce [11]. In spite of this, more than 150 cases of infection were reported in tropical and subtropical regions, including India, where C. violaceum is normally found [12]. This rare infection is associated with a high mortality rate, between 60% and 80%, if not diagnosed at an early stage or treated correctly [11]. A recent
case of a man from South India with septicemic \textit{C. violaceum} infection and septic arthritis, who had a fatal outcome, was reported [13]. A likely explanation for the high mortality rate could be the resistance of \textit{C. violaceum} to a wide range of antibiotics and to other mechanisms that pump out the cytotoxic drugs [14]. Thus, appropriate therapy is absolutely essential to control this neglected, potentially fatal infection. The strategy of controlling pathogens by interrupting its QS phenomenon was the prime focus of research in the recent years. The importance of quorum sensing in \textit{C. violaceum} pathogenesis was demonstrated by the fact that QS-antagonist molecules protect the nematode \textit{Caenorhabditis elegans} from \textit{C. violaceum}-mediated killing [15]. The \textit{C. violaceum} quorum-sensing system consists of the LuxI/LuxR homologues CivI/CivR, which controls virulence and the production of a variety of phenotypic characteristics that includes the production of the purple pigment, violacein, cytolytic and the antibiotic phenazine. The complete genomic sequence of \textit{C. violaceum} ATCC 12472 has also revealed the presence of these QS-associated genes [16].

Inhibition of QS by some chemically synthesized compounds was identified but most of the QS inhibitors were isolated and characterized from plant sources [17,18]. Crude extracts of many plant parts were shown to possess anti-QS activity using \textit{C. violaceum} as a model bacterium [19,20,21]. Crude plant extracts are often found to be more effective than isolated constituents at an equivalent dose perhaps owing to positive interactions between components of whole plant extracts. This synergy may involve prevention of the active component from degradation by enzymes or facilitate transport across cell barriers that result in higher efficacy of the crude drug when compared with purified components [22,23]. Hence, it is lately realized that crude extracts may possibly be the right strategy to treat multi-drug resistant pathogens as compared to the purified compound isolated from the same extract. In fact, the use of traditional herbal medicines is sometimes considered more effective than conventional drugs for the treatment of disease such as malaria [24]. It is proposed that the new generation of phytopharmaceuticals may enable successful use of herbal drug combinations to treat diseases in comparison to single active component [25].

\textit{Psidium guajava} L. (Guava), widely distributed throughout India, belongs to the family Myrtaceae and is a well known traditional medicinal plant widely used in folk medicine [26,27]. The leaf extracts of this plant were shown to possess anti-microbial [28], anti-inflammatory, antidiarrhoea [29], anti-oxidant [30], antimutagenic [31], anti-cancer [32], anti-diabetic [33] and anti-plaque [34] activities. However, no molecular mechanism of antimicrobial property of guava-leaf extract was explored.

To the best of our knowledge, this is the first attempt to reveal gene expression profile of \textit{C. violaceum} with the aid of whole transcriptome analyses on Ion-Torrent in presence of guava-leaf extract (GLE). GLE inhibited QS-controlled genes and QS-regulated phenotypes without affecting the bacterial growth up to 24 h suggesting these effects to be unrelated to bacteriostatic or bactericidal effects. Furthermore, the down-regulation of the wide array of genes, including those encoding virulence factors, affect pathogenicity as revealed by the ability of GLE to arrest \textit{C. violaceum} induced cell lysis of human hepatoma cells.

**Materials and Methods**

**Bacterial strains**

The \textit{Chromobacterium violaceum} wild type strain MTCC 2656 and \textit{Pseudomonas aeruginosa} MTCC 2297 were obtained from the Microbial Type Culture Collection Center (MTCC), IMTECH, Chandigarh, India. MTCC 2656 and MTCC 2297 cells were routinely cultured on Nutrient broth (NB, Hi-Media- M002) agar and Luria-Broth (LB, Hi-Media-M575) agar respectively and maintained at 37°C.

**Extraction of guava leaves**

Leaves of \textit{Psidium guajava} L. (Guava) were collected from the Centre of Floriculture and Agro Business Management (COFAM), University of North Bengal and extracted following standard method [35]. The leaves were washed thoroughly with sterile distilled water and rinsed with 70% (v/v) ethanol. Washed leaves were dried under sun initially and finally in the oven at 50°C for 1 hour. The dried leaves were crushed to fine powder, passed through an 80 mesh sieve and stored in a sealed plastic bag. To 50 gm of powder, 500 ml of sterile distilled water was added and the mixture was heated at 70°C for 1 hour and incubated at 30°C for 72 hours with shaking at regular intervals. The extract was filtered through a Whatman No. 1 filter paper (pore size 11 μm) and centrifuged at 10,000 rpm for 10 min. The resultant supernatant was freeze-dried using a lyophilizer (IIC Industrial Corporation, India). The dried sample was reconstituted with water, filter-sterilized (0.45 μm pore size, Sartorius Stedim, Germany) and tested for its ability to modulate quorum sensing in \textit{Chromobacterium violaceum} strain MTCC 2656.

**Demonstration of Inhibitory effect of Guava leaf extract (GLE) on Quorum Sensing (QS) activity of \textit{C. violaceum} MTCC 2656**

The inhibition of QS-mediated violacein production in \textit{C. violaceum}, by GLE, was studied by the agar well assay [36]. Sterile molten Nutrient agar (Hi-Media, India) was pour- plated with the cells of \textit{C. violaceum} MTCC 2656. After solidification of the plates, wells were made in which 100 μl of overnight grown MTCC 2297 was supplemented with 5% glucose and 0.5% agar containing either water (control) or 400 μg ml⁻¹ of GLE was poured in petri dishes. An inoculum of 10 μl of overnight grown MTCC 2297 was inoculated at the center of the plates. The inoculated plates were

---

**Figure 1. Effect of GLE on violacein production and growth of wild-type \textit{C. violaceum} MTCC 2656 cells.** A. Violacein production in the presence of different concentrations of GLE. B. Viable cell number in batch culture grown without (Control) or with (Experimental) supplementation of 400 μg ml⁻¹ GLE. C. Quantitation of violacein in batch culture grown without (Control) or with (Experimental) supplementation of 400 μg ml⁻¹ GLE.

doi:10.1371/journal.pone.0107703.g001
incubated at 37°C for 20 h and motility across the agar surface was visualized.

Determination of QS-inhibitory concentration of GLE with reference to violacein production

Log phase cells of MTCC 2656 (2.5×10^6 CFU ml^{-1}) were inoculated (1.0% inocula) into 10 ml volumes of sterile nutrient broth in flasks containing different concentrations of the GLE (100 μg ml^{-1} to 1000 μg ml^{-1}) and incubated at 37°C for 24 h with shaking. The control set was devoid of GLE. The violacein pigment formation in the flask was quantified following the method of Choo et al. [38]. Briefly, 1 ml culture from each flask was centrifuged at 13,000 rpm for 10 min. The culture supernatant was discarded and 1 ml of DMSO was added to the pellet. The solution was vortexed vigorously to completely solubilize violacein and centrifuged at 10,000 rpm for 10 min to remove the cells. The absorbance of the supernatant was read at a wavelength of 585 nm in a digital spectrophotometer (ThermoSpectronic UV). The maximum O.D._{585} value observed in case of GLE-untreated cells was considered as 100% production of violacein.

Figure 2. Profile of genome-wide gene expression in *C. violaceum* MTCC 2656 cells. Cells were grown in presence (experimental) and absence of 400 μg ml^{-1} GLE (control).

doi:10.1371/journal.pone.0107703.g002

Figure 3. Differential expression of genes in *C. violaceum* in presence of GLE. The classification was based on Clusters of Orthologous Groups (COG) functional classification (R, General function prediction only; S, Function unknown; and NRF, No results found).

doi:10.1371/journal.pone.0107703.g003
**Table 1.** Significantly downregulated *C. violaceum* genes associated with quorum-sensing and pathogenicity, in presence of GLE.

| ORF no. | Gene name | Description | Control | Expt | \( \log_{2}(\text{fold change}) \) | p value |
|---------|-----------|-------------|---------|------|-----------------|---------|
| **Constituent of Quorum sensing circuit** | | | | | |
| CV_4091 | cviI | N-acyl homoserine synthase; autoinducer synthase, quorum controlled system | 171.85 | 19.44 | 3.14 | 5.39E-005 |
| CV_4090 | cvrR | transcriptional activator, LuxR/UhpA family of regulators | 194.51 | 14.75 | 3.72 | 3.04E-006 |
| **Genes predicted to have cvrR binding sites** | | | | | |
| CV_1311 | hypothetical | | 46.41 | 1.6 | 4.86 | 0 |
| CV_1329 | sbcB | exonuclease I | 15.82 | 0.66 | 4.59 | 0 |
| CV_1328 | methyl-accepting chemotaxis protein | | 39.91 | 2.86 | 3.8 | 3.86E-006 |
| CV_1408 | sdaA2 | serine dehydratase | 23.25 | 2.67 | 3.12 | 0 |
| CV_1444 | hypothetical | | 14.81 | 0.94 | 3.98 | 0.01 |
| CV_2091 | putative tetR-family transcriptional regulator | | 23.69 | 2.89 | 3.03 | 0.01 |
| CV_2321 | recN | DNA repair protein | 19.88 | 0.54 | 5.19 | 7.18E-006 |
| CV_2434 | hypothetical | | 56.1 | 1.93 | 4.86 | 0 |
| CV_2656 | cytochrome P450 hydroxylase | | 96.94 | 8.63 | 3.49 | 4.07E-005 |
| CV_2716 | hypothetical | | 52.45 | 5.47 | 3.26 | 4.07E-005 |
| CV_3062 | enoyl-CoA hydratase | | 32.72 | 1.31 | 4.64 | 0 |
| CV_3300 | treB | protein phosphohistidine-sugar phosphotransferase | 8.82 | 0.55 | 4 | 0 |
| CV_4091 | cviI | autoinducer synthase | 171.85 | 19.44 | 3.14 | 5.39E-005 |
| CV_4092 | aldehyde dehydrogenase | | 20.7 | 1.39 | 3.9 | 0 |
| CV_4142 | hoxX | HoxX-like protein | 7.99 | 0.53 | 3.9 | 0.01 |
| CV_4240 | putative chitinase | | 41.52 | 2.82 | 3.92 | 2.08E-006 |
| CV_4379 | hypothetical inside biotin synthesis operon | | 16.18 | 0.61 | 4.74 | 1.65E-005 |
| CV_4382 | comF | competence protein F | 26.97 | 1.71 | 3.98 | 0.01 |
| **Quorum Sensing controlled genes** | | | | | |
| CV_1682 | hcnC | hydrogen cyanide synthase HcnC | 187.54 | 6.8 | 4.78 | 7.43E-009 |
| CV_1683 | hcnB | hydrogen cyanide synthase HcnB | 600.99 | 4.65 | 7.01 | 0 |
| CV_1684 | hcnA | hydrogen cyanide synthase HcnA | 600.99 | 4.65 | 7.01 | 0 |
| **Protease** | | | | | |
| CV_2571 | LasA | LasA protease precursor | 175.34 | 13.11 | 3.74 | 1.13E-006 |
| CV_2571 | LasB | class 4 metalloprotease | 211.37 | 20.93 | 3.34 | 1.09E-006 |
| **Cellulose biosynthesis** | | | | | |
| CV_2675 | bscC | cellulose synthase, subunit C | 9.3 | 0.22 | 5.39 | 0 |
| CV_2676 | bscZ | endo-1,4-D-glucanase | 9.3 | 0.22 | 5.39 | 0 |
| CV_2677 | bcsB | cellulose synthase, subunit B | 9.3 | 0.22 | 5.39 | 0 |
| **Other genes related to virulence and pathogenicity** | | | | | |
| CV_3824 | conserved hypothetical protein | | 64.28 | 5.74 | 3.49 | 6.81E-005 |
| CV_3828 | pilB | type 4 fimbrial biogenesis protein | 63.95 | 5.32 | 3.59 | 5.99E-006 |
| CV_0829 | pilQ | type 4 fimbrial biogenesis protein PilQ | 96.29 | 2.11 | 5.51 | 1.01E-013 |
| CV_0830 | pilP | type 4 fimbrial biogenesis protein PilP | 96.39 | 2.11 | 5.51 | 1.01E-013 |
| CV_0832 | pilN | type 4 fimbrial biogenesis protein PilN | 85.03 | 6.41 | 3.73 | 0 |
| CV_0833 | pilM | type 4 fimbrial biogenesis protein PilM | 130.37 | 15.8 | 3.04 | 5.79E-005 |
| CV_0179 | pilT | twitching motility protein PilT | 53.97 | 6.7 | 3.01 | 0 |
| CV_0180 | pilU2 | twitching motility protein transport | 32.64 | 2.57 | 3.67 | 0 |
| CV_1458 | pilUI | twitching motility protein | 41.43 | 5.17 | 3.0 | 0 |
| CV_3112 | pilV | type-4 fimbrial biogenesis PilV transmembrane protein | 79.2 | 2.24 | 5.14 | 0 |
| CV_2618 | sipC | cell invasion protein | 17.62 | 0.92 | 4.25 | 0.01 |
| CV_2619 | sipB | cell invasion protein | 116.65 | 3.16 | 5.21 | 6.23E-005 |
Table 1. Cont.

| ORF no. | Gene name | Description | Control | Expt | log2 (fold change) | p value |
|---------|-----------|-------------|---------|------|-------------------|---------|
| CV_2620 | spaT      | surface presentation of antigens; secretory proteins | 116.65  | 3.16 | –5.21            | 6.23E-005 |
| CV_2198 | pyrH      | uridyliate kinase, Pyrimidine metabolism | 40.96  | 4.63 | –3.15            | 0       |
| CV_2205 | ampH      | hypothetical protein, outer membrane protein | 46.62  | 5.62 | –3.05            | 0.01    |
| CV_2206 | lpxO      | Lipopolysaccharide biosynthesis | 41.56  | 4.12 | –3.33            | 0.01    |
| CV_2207 | fabZ      | 3-hydroxyacyl-[acyl-carrier-protein] dehydratase, Fatty acid biosynthesis | 41.56  | 4.12 | –3.33            | 0.01    |
| CV_2209 | lpxB      | lipid-A-disaccharide synthase, Lipopolysaccharide biosynthesis | 29.44  | 3.69 | –3               | 0       |
| CV_2210 | mhlB      | ribonuclease HII | 29.44  | 3.69 | –3               | 0       |
| CV_2212 | hypothetical protein | 561.06 | 41.05 | –3.77 | 0               |
| CV_4338 | ftsZ      | cell division protein | 61.92  | 7.3  | –3.08            | 9.58E-005 |
| CV_4340 | ftsQ      | cell division transmembrane protein | 41.07  | 1.13 | –5.19            | 0       |
| CV_4341 | ddlB      | D-alanine-D-alanine ligase, Peptidoglycan biosynthesis | 41.07  | 1.13 | –5.19            | 0       |
| CV_4344 | ftsW      | cell division protein | 25.93  | 3.05 | –3.09            | 0       |
| CV_4345 | mraY      | Peptidoglycan biosynthesis | 25.93  | 3.05 | –3.09            | 0       |
| CV_4346 | mraY      | Peptidoglycan biosynthesis | 19.51  | 1.81 | –3.43            | 0       |
| CV_4349 | ftsI      | cell division protein FtsI | 157.11 | 0.52 | –8.25            | 3.40E-008 |
| CV_4350 | ftsI      | cell division protein | 157.11 | 0.52 | –8.25            | 3.40E-008 |
| CV_4351 | mraW      | 16 S rRNA (cytosine1402-N4)-methyltransferase | 157.11 | 0.52 | –8.25            | 3.40E-008 |
| CV_4352 | conserved hypothetical protein | 157.11 | 0.52 | –8.25 | 3.40E-008 |
| CV_0516 | Ca binding hemolysin | 7.43   | 0.71  | –3.39            | 3.36E-005 |
| CV_0360 | thermolabile hemolysin | 25.86  | 2.28  | –3.5             | 0       |
| CV_1989 | porin     | 257.04  | 24.41 | –3.4             | 6.76E-006 |
| CV_3104 | porin     | 1207.93 | 95.97 | –3.65            | 7.68E-006 |
| CV_3829 | porin     | 813.08  | 69.81  | –3.54            | 6.04E-006 |
| CV_3342 | hemolysin III | 274    | 26.88 | –3.35            | 1.39E-005 |

1gene expression in cells grown for 24 h.
2gene expression in cells grown for 24 h in presence of 400 μg ml⁻¹ of GLE.
doi:10.1371/journal.pone.0107703.t001

The percentage (%) inhibition in violacein production was calculated as follows: % inhibition in violacein production = [(O.D.585 value observed in the absence of GLE – O.D.585 value observed in presence of a defined quantity of GLE)/O.D.585 value observed in absence of GLE]×100.

Determination of growth and violacein production of C. violaceum MTCC 2656 in presence and absence of GLE

Growth of MTCC 2656 was quantified in terms of viable cell number present in the culture (dilution plating followed by counting CFUs) at different time interval [39]. Briefly, an overnight culture of MTCC 2656 (in NB medium), that had been inoculated with a freshly grown single colony, was diluted 10-fold into 10 ml NB medium and allowed to grow for 4 h to obtain log phase cells. The culture was then inoculated into 800 ml NB medium and divided into two portions, to one of which water (control) and to the other, GLE at a final concentration of 400 μg ml⁻¹, was added. These were finally distributed in 10 ml precultivated 100 ml Erlenmeyer flask, and incubated at 37°C with shaking. At different time intervals, cultures were withdrawn from time-defined flasks (both control and test) for dilution plating onto NB agar plates as well as for the estimation of violacein by the method described above.

Whole genome transcriptome analysis

The detail of the methodology is provided in File S1. In brief, RNA was isolated from control (cells grown without GLE) and experiment (cells grown with GLE) samples and cDNA Library was prepared using the Ion Total RNA-Seq Kit v2 (Catalog Number 4475936). Template preparation and enrichment was performed as per Ion OneTouch 200 Template Kit (Cat no. 4471263) and 200 base-read sequencing was performed using the Ion PGM 200 Sequencing Kit (Cat no 4474004) on ION TORRENT.

Infection and morphological assessment of HepG2 cells by phase-contrast inverted microscope

HepG2 cells (Human hepatocellular liver carcinoma cell line), obtained from Cell Culture Collection, NCCS, Pune, were grown in 100 mm polyvinyl coated plates, in DMEM (Dulbeco’s Modified Eagle’s Medium) media with 10% FCS at 37°C in a humidified atmosphere containing 5% CO₂. Cells (1×10⁶) were seeded in 60 mm plates in DMEM medium supplemented with 10 U/ml penicillinG and 100 μg ml⁻¹ streptomycin. After 24 h, the media was removed and cells were washed thrice with phosphate buffer (PBS, pH 7.2) and grown in DMEM media without antibiotics until C. violaceum infection. Bacteria (MTCC 2656) were grown overnight at 37°C in NB either without or with...
The following day, the bacterial cells were grown further in fresh media under the same conditions for 3 h. Finally, the untreated or GLE treated *C. violaceum* cells were diluted in DMEM with 10% FCS supplemented either with water or GLE, and added to HepG2 cells at the infectivity ratio of 10:1. The plates were incubated at 37°C in an atmosphere of 5% CO₂ and observed under inverted microscope (Olympus, ck40-slp) at 0 and 4 h of infection at 200X magnification and photographed. HepG2 cells incubated in DMEM containing GLE served as control.

**LDH cytotoxicity assay**

Cytotoxicity induced in HepG2 cells was quantitated by measuring the release of the cytosolic enzyme lactate dehydrogenase (LDH) in the culture medium. For this, HepG2 cells were grown and exposed to *C. violaceum* as described above. After 4 h of incubation, supernatants were collected and evaluated for the presence of LDH using the LDH Cytotoxicity Assay Kit (Item No. 10008882, Cayman Chemical Company). The LDH activity (µU) was determined from the standard curve and the total LDH activity (µU ml⁻¹) was calculated as value from LDH activity assay (µU)/sample volume assayed (ml).

**Statistical Analysis**

All experiments were performed in triplicate. Results are expressed as mean value ± standard deviations (S.D) of three replicates and analyzed by using the software SPSS 15.0 for windows (SPSS Inc. Chicago, II, USA). The statistical treatment of “Whole Transcriptome Analysis” data is elucidated in File S1.
Results and Discussion

Inhibition of violacein production in the presence of aqueous extracts of guava leaves

The best studied trait controlled by QS in C. violaceum is the production of the purple pigment, violacein [40]. To investigate QS inhibitory activity of aqueous extract of Psidium guajava L. leaves (GLE), its effect on violacein synthesis by C. violaceum MTCC 2656 was examined. A colourless transluscent zone around the zone of diffusion of GLE from the agar cup indicated growth of C. violaceum failing to produce violacein (purple pigment, Figure S1A). The production of violacein pigment at 24 h in the presence of varied concentration of GLE was quantified spectrophotometrically (Figure 1A). Violacein production was inhibited by 85% in presence of 400 μg ml⁻¹ of GLE and the extent of inhibition remained similar at concentrations up to 1000 μg ml⁻¹. Thus, the concentration of GLE to be used for further studies was selected as 400 μg ml⁻¹. It has been documented in the Literature that nearly all parts of Psidium guajava L. tree, including fruits, leaves, bark, and roots, were used traditionally as a medicinal plant throughout the world for varied ailments [27]. The present study revealed that the guava-leaf extract was capable of inhibiting the quorum-dependent violacein production in C. violaceum.

The growth of C. violaceum is unaffected in presence of GLE

The number of viable cell enumerated from growth studies in presence and absence of the GLE (400 μg/ml) showed no significant difference (Figure 1B). On the contrary, the violacein production was significantly inhibited under similar growth conditions (Figure 1C). It has been noted by earlier authors that the toxicity of putative quorum sensing inhibitors towards bacterial cells may be assessed by addressing three principal issues; that the QS inhibitory effects occur below the minimum inhibitory concentration (if the inhibitor exhibits antimicrobial property), the quorum-sensing inhibitory concentration used in the study does not affect the final cell density after a certain period of incubation, and the QS inhibitor does not grossly affect the kinetics of growth [41]. Our results show that the growth of C. violaceum remained unaffected but the quorum-dependent production of violacein was significantly reduced in the presence of GLE. These observations led us to conclude that the decrease in violacein production was not due to any form of growth inhibition of the cells in the culture medium at least up to 24 h of incubation. Similar results with respect to concentration-dependent decrease in violacein production without affecting bacterial growth were reported with extracts of various medicinal plants [42,43]. Assuming that the quorum-inhibiting property of GLE should also be yielding in other bacteria that demonstrate quorum-dependent diverse phenotypes, we tested GLE for its activity using another test organism, Pseudomonas aeruginosa. The gram-negative bacterium Pseudomonas aeruginosa exhibits swarming motility which requires the bacteria to effectively work together via the process of quorum-sensing [37]. The cells of P. aeruginosa, MTCC 2297, in absence of GLE, formed tendrils migrating outwards from the point of bacterial inoculation, with continued branching as the bacteria moved farther from the center, while in presence of GLE, cells grew to form a localized colony in the center with no signs of swarming (Figure S1B). The ability of natural products to disrupt the quorum regulated swarming motility in P. aeruginosa was demonstrated earlier [44]. Thus, the manifestation of anti-QS property in the form of inhibition of swarming motility of P. aeruginosa, further provides evidence for the proposed quorum quenching property of GLE.

Impact of GLE on the genome-wide gene expression of C. violaceum

The quorum-regulated gene expression has been extensively studied in gram negative pathogenic bacteria such as P. aeruginosa [45] and Escherichia coli [46]. However, C. violaceum transcriptomic studies have not yet attracted the similar attention.
The increasing reports of *C. violaceum* cases [47] prompted us to unravel the status of gene expression in the presence or absence of GLE with a view to establish GLE as a QS inhibitory candidate for controlling the bacterial pathogenicity. Schuster *et al.* have observed that the timing affects the quorum-controlled gene expression in *P. aeruginosa* and most of the transcripts under QS-regulation were induced maximally at late log or stationary phase [48]. Thus, to get an insight into the effect of GLE on global gene expression of *C. violaceum*, we used 24 h grown cultures either in the absence (Control) or presence (Experimental) of 400 μg ml⁻¹ GLE.

A total of 33,54,744 and 30,09,475 high quality Ion Torrent reads for Control and Experiment sample respectively were mapped on the reference genome of *Chromobacterium violaceum* ATCC 12472 with genome size of 4.75 Mb. There are 4,529 genes present in *C. violaceum* ATCC 12472 GFF file. The number of genes expressed in (i) both control and experiment; (ii) exclusively in control; (iii) exclusively in experiment; (iv) not expressed in both control and experiment were determined to be 3025, 1229, 32, and 243 respectively, as illustrated in Figure 2. Raw sequences were deposited at the NCBI Sequence Read Archive, under Bioproject accession number PRJNA243990 and SRA accession code SRP041018 [http://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA243990]. The FPKM value for each gene was calculated for the combination of samples Control and Experiment. These FPKM values (FPKM_control and FPKM_experiment) were further used to calculate the log fold change as log₂ (FPKM_experiment/FPKM_control). Moreover, uncorrected p-value of the test statistic for each gene was also computed. Using a stringent log₂ threefold cut-off with p-value≤0.01, we observed that 816 genes, scattered throughout the *C. violaceum* MTCC 2656 genome, comprising about 19% of the total genes expressed, were significantly down regulated in the presence of GLE (Table S1). The 816 differentially expressed genes were categorised into 17 groups (Figure 3) based on the *C. violaceum* ATCC12472 database from the Brazilian Genome Virtual Institute of Genomic Research (BRGene) [http://www.brgene.lncc.br]. The category representing the largest percentage, ~32% of the total 816 genes, encoded for the group of proteins assigned unclassified and unknown functions. The categories representing >6.5% belonged to the functional classes of proteins namely cell motility and secretion (9.31%), cell envelope biogenesis, outer membrane (7.47%), transcription mechanisms (6.86%) and amino acid transport and metabolism (8.94%). Among these, the first three groups of proteins are related to virulence in many pathogens since these processes mediate interaction of bacteria with their immediate environment and genes encoding virulence factors. GLE down-regulated genes like lyases (*argH, aspA*), dehydrogenase and hydratase (*usg, sdaA*) and transporters (*sdaC, argT*), that encode products involved in metabolism and transport of amino acids. In addition, GLE down-regulated, about 4%, the genes involved in energy production and conversion, secondary metabolites biosynthesis, inorganic ion transport and metabolism, and coenzyme metabolism. The possibility of interference of GLE with one or several global regulators in *C. violaceum* resulting in differential expression of multiple genes cannot be ruled out. However, since GLE targeted virulent genes which are often QS controlled [49] and QS activated genes involved in uptake, synthesis and degradation of amino acids [50]; inhibited QS controlled phenotype, violacein production, without hampering growth of *C. violaceum* up to 24 h, pointed to the possible anti-QS property of GLE. Repression of genes related to cellular processes with no effect on growth was revealed by microarray analysis of *P. aeruginosa* gene expression in the presence of QS inhibitor furanone C-30 [51]. Earlier microarray studies with *E. coli* and *P. aeruginosa* have revealed that varied cellular functions are largely affected by the QS system [45,46]. Both the gram negative bacteria have more than one QS regulatory system. The cross-talk between different quorum signal pathways lead to either synergistic or antagonistic effects. It is thus tempting to assume that *C. violaceum* may also possess multiple QS systems and any interference would thus lead to a global differential expression of genes as observed with GLE. Previous transcriptomic studies have shown that as much as >10% of the total genes in the genome of *P. aeruginosa* are QS regulated [45], however, we observed a higher percentage (19%) of differential gene expression. This could be explained either due to the presence of multiple components of QS or if we assume that these genes are QS-regulated, the higher number of genes revealed in this study may be due to the fact that earlier groups detected QS regulators using specific mutants but our studies involves revelation of QS regulators using a putative QS inhibitor on wild type cells. Thus, although the data obtained does not reflect a definitive analysis of gene regulation by quorum sensing, perhaps for the first time, our results provide with a set of probable candidate genes under QS regulation in *C. violaceum* for further investigation.

**Differential expression of genes predicted to be under QS-regulation in *C. violaceum***

Cell to cell signaling in *C. violaceum* is based on the CviI/CviR circuit. At high cell density, the cytoplasmatic quorum-sensing receptor, CviR, binds to the AHL autoinducer. The CviR-AHL complex then binds to DNA and activates transcription of a number of genes. The fact that GLE has the ability to induce break in the CviI/CviR circuitry is reflected phenotypically by the abolition of violacein production by *C. violaceum* in the growth medium. The gene responsible for violacein production, *violA*, was log₂/threefold repressed. Since the level of expression of *violA* gene (FPKM_control value) in absence of GLE was low (perhaps owing to the experimental conditions), the comparison with FPKM_experiment value (in presence of GLE) was concluded as statistically non-significant. Our data revealed that GLE down-regulated the expression of major virulence factors (Table 1) such as, *lasA* (CV_2571), *lasB* (CV_0057), *hcnABC* operon (CV_1682 to CV_1684) and chitinase (CV_4240). The quorum regulated proteases LasA and LasB are capable of inactivating a wide range of biological tissues and immunological agents to allow the bacteria to invade and colonize host tissues [52,53]. The *hcnABC* operon responsible for biosynthesis of hydrogen cyanide (HCN), a secondary metabolite and a potent inhibitor of cytochrome oxidase and several other metalloenzymes, is known to be under the control of quorum-sensing mechanism in *C. violaceum* [54]. The enzyme, chitinase, is hypothesized to be involved in blocking the growth of fungi with chitinous exoskeleton, allowing a competitive advantage to *C. violaceum* during colonization [55]. In addition to these secreted factors, transcription of gene encoding acyl carrier protein (ACP) synthase, *fabF* (CV_3412) and other ACP-encoding genes like *fabZ* (CV_2207) and *CV_3413*, were significantly repressed by GLE. These gene products are proposed to be involved in synthesis of AHLs [56]. Similar down-regulation of these genes in *P. aeruginosa* was observed in the presence of the QS inhibitor, furanone C-30 [51]. Moreover, C-30 also targets *P. aeruginosa* virulence but it does not affect the expression of gene clusters encoding the components of QS system. On the contrary, GLE not only affected virulence but significantly repressed the expression of both *cviI* (CV_4091) and *cviR* (CV_4090) genes, the basic constituents of *C. violaceum* QS system, suggesting direct transcriptional interference of GLE.
A putative cviR binding site was detected in the cviI promoter region and so its expression was expected to be down-regulated. In addition, a number of other cviR-regulated genes based on the presence of an ideal CvIR binding site coupled with genome scanning were predicted [57]. GLE down-regulated a subset of these genes, which included CV_1320 (methyl-acceptor chemotaxis protein), CV_2291 (recA, DNA repair protein) and CV_3062 (enoyl-CoA hydratase). The down-regulation of QS-controlled genes with no effect on bacterial cell growth led us to hypothesize the anti-QS property of GLE to be responsible for the observed differential gene expression.

**Status of expression of genes plausibly networked with quorum sensing mechanism**

The pathogenicity of *C. violaceum* is still poorly understood. Cause and effect relationship between QS system and pathogenicity of *C. violaceum* is yet an enigma. On the basis of whole genome sequence of *C. violaceum*, a catalogue of genes encoding probable virulence factors was prepared [58]. These included the genes encoding pil proteins and genes involved in lipopolysaccharide (LPS) and peptidoglycan biosynthesis. The type IV pilus gene cluster encodes Type IV pil which are appendages emanating from the surfaces of several gram-negative bacteria that are associated with pathogenicity [59,60]. The expression of a majority of the pil genes found in *C. violaceum*, namely pil B, T, U2, U1, V and pilQPNM, were inhibited by GLE. Transcription of lpxB (CV_2299) and lpxD (CV_2296) genes, responsible for LPS biosynthesis and murA (CV_0440) and murD (CV_4345), genes required for peptidoglycan biosynthesis, were significantly down-regulated in the presence of GLE. As found in other gram negative bacteria, the LPS and peptidoglycan of *C. violaceum* are associated with activation of immune response in the host, which induces secretion of inflammatory cytokines resulting in septic shock [58]. Very recently, genes encoding potential secreted virulence factors of *C. violaceum* were identified [61]. Among these, GLE targeted the hemolysins (CV_0516, CV_0360 and CV_3342) and porins (CV_1989, CV_3104, and CV_3829). Increased expression of hemolysin and porins in presence of quorum signal autoinducer 2 (AI-2), mediator of second signaling pathway, has previously been documented in *E. coli* [46]. While unravelling the virulence determinant for *C. violaceum* induced cell lysis, Miki *et al.* observed that the formation of pore structures on the host cell membrane results in cell death in hepatocytes [62]. The pore formation involved the release from the host cell (Figure 5). The guava leaf paste was highly likely to be toxic towards the non-infected cells (Figure 5). Interestingly, the amount of LDH increase in extracellular LDH activity was observed as compared with subsequent release of LDH [62]. In agreement, after 4 h following *C. violaceum* infection in HepG2 cells, a significant increase in extracellular LDH activity was observed as compared to the non-infected cells (Fig. 5). Interestingly, the amount of LDH release from *C. violaceum*-infected HepG2 cells decreased by ~80% in the presence of GLE indicating the ability of GLE to hinder cell death instigated by *C. violaceum*. A similar decrease in cytotoxicity in different cell lines was demonstrated using mutant strains of *C. violaceum* [62]. Moreover, GLE itself had no effect on human hepatoma HepG2 cells showing its non-toxicity towards the host cell (Figure 5). The guava leaf paste was highly likely to be non-toxic since it is used immensely in folklore practices, including as toothpaste for the cure of dental caries [70]. This result shows that GLE inhibits *C. violaceum*-induced cytotoxicity in HepG2 cells.

**GLE inhibits *C. violaceum*-induced cytotoxicity in HepG2 cells**

To analyze the effect of GLE on cell death induced by *C. violaceum*, human hepatocellular liver carcinoma cell line HepG2 was infected with the bacteria either in the absence or presence of GLE. The course of infection was monitored by phase contrast microscopy at different time points. It was observed that *C. violaceum* was able to induce cytotoxicity in HepG2 cells, characterized by shrinkage of the cells and reduction in cell density, with time (Figure 4A). Whereas, no morphological changes were observed in the cells when infection was carried out in presence of GLE indicating that the extract could protect the HepG2 cells from *C. violaceum* infection (Figure 4B). Notably, GLE alone had no inhibitory effect on the growth of HepG2 cells (Figure 4C). Furthermore, *C. violaceum*-induced cytotoxicity was quantified by assaying the release of the cytosolic enzyme lactate dehydrogenase (LDH). The cell death observed after *C. violaceum* infection in hepatocytes is characterized by membrane rupture with subsequent release of LDH [62]. In agreement, after 4 h following *C. violaceum* infection of HepG2 cells, a significant increase in extracellular LDH activity was observed as compared to the non-infected cells (Fig. 5). Interestingly, the amount of LDH release from *C. violaceum*-infected HepG2 cells decreased by ~80% in the presence of GLE indicating the ability of GLE to hinder cell death instigated by *C. violaceum*. A similar decrease in cytotoxicity in different cell lines was demonstrated using mutant strains of *C. violaceum* [62]. Moreover, GLE itself had no effect on human hepatoma HepG2 cells showing its non-toxicity towards the host cell (Figure 5). The guava leaf paste was highly likely to be non-toxic since it is used immensely in folklore practices, including as toothpaste for the cure of dental caries [70]. This result shows that GLE inhibits the pathogenicity of *C. violaceum* by preventing the initiation of the cascade of gene expression required for successful infection and establishment in the host.
which, in turn, may provide time to the immune system of the host to eliminate the pathogen. This effect of GLE may be attributed to its ability to either directly or indirectly inhibit the QS system.

Conclusions

Identification of QS inhibitors (QSIs) from natural products as an alternative to antibiotics is currently an area of intense interest. However, establishment of a real QSI and the phenomenon of resistance to QSIs are debatable aspects in this field of research [71]. The present study proposes guava leaf aqueous extract (GLE) as QSI since it inhibits quorum regulated phenotypes such as violacin production and swarming in the pathogenic bacteria C. violaceum and P. aeruginosa respectively and induces a global differential gene expression in C. violaceum, a pathogen with high mortality rate, without affecting its growth. The complexity of GLE prevents us from directly linking it with the QS system. However, reduction in QS gene expression was correlated to the attenuation of bacterial virulence resulting in prevention of C. violaceum induced lysis of host (HepG2) cells in vitro. More studies are required to establish the exact nature of the metabolic cross-talks in presence of GLE but this study provides with the platform to think on using the crude extract of plants to combat pathogenic bacteria. Overall, our results provide insights into the candidature of GLE as QSI and the identification of a set of probable quorum regulated genes in C. violaceum.

Supporting Information

Figure S1 Inhibition of QS-regulated phenotypes by GLE. A. Formation of a colourless translucent zone around the well containing GLE indicating absence of violacein production by C. violaceum cells. B. Inhibition of swarming motility of P. aeruginosa cells grown in presence of GLE. (TIF)

Table S1 Significantly down-regulated genes of C. violaceum when grown in presence of GLE. (DOCX)

File S1 Method: Whole genome transcriptome analysis. (DOCX)

Author Contributions

Conceived and designed the experiments: RG RC. Performed the experiments: BKT AK. Analyzed the data: RG RC. Contributed reagents/materials/analysis tools: RG RC BKT AK. Contributed to the writing of the manuscript: RG RC BKT AK.

References

1. Rasmussen TB, Givkov M (2006) Quorum-sensing inhibitors as anti-pathogenic drugs. Intl J Med Microbiol 296: 149–161.
2. Clatworthy AE, Pierson E, Hung DT (2007) Targeting virulence: a new paradigm for antimicrobial therapy. Nat Chem Biol 3: 541–548.
3. Kalia VC, Purushot HJ (2011) Quenching the quorum sensing system: potential antibacterial drug targets. Crit Rev Microbiol 37: 121–140.
4. Williams P (2007) Quorum sensing, communication and cross-kingdom signalling in the bacterial world. Microbiology 153: 3923–3938.
5. Sarkar S, Chakraborty R (2008) Quorum sensing in metal tolerance of Acinetobacter baylyi ADP1 is associated with biofilm production. FEMS Microbiol. Lett 282: 160–165.
6. Dandekar AA, Chugani S, Greenberg EP (2012) Bacterial quorum sensing and metabolic incentives to cooperate. Science 330: 264–266.
7. Waters CM, Bassler BL (2005) Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol 21: 319–346.
8. Schuster M, Sexton DJ, Diggle SP, Greenberg EP (2013) Acyl-homoserine lactone quorum sensing: From evolution to application. Annu Rev Microbiol 67: 43–63.
9. Rutherford ST, Basler BL (2012) Bacterial quorum sensing: Its role in virulence and possibilities for its control. Cold Spring Harb Perspect Med 2: a01242.
10. Koburger JA, May SO (1982) Isolation of Chromobacterium spp. from foods, soil, and water. Appl Environ Microbiol 44: 1463–1465.
11. Orsetti AG, Markiewicz P, Epstein MG, Conceição OJG, D’Epolito G, et al. (2013) Liver abscesses by Chromobacterium violaceum: a case report of a rare disease. OA Case Reports 20: 19–23.
12. Tosh AYB, Hui M, Ngo KY, Wong J, Lee KF, et al. (2006) Fatal septicaemia from Chromobacterium violaceum: Case reports and review of the literature. Hong Kong Med J 12: 228–231.
13. Karthik R, Rancharatanam P, Balaji V (2012) Case Report Fatal Chromobacterium violaceum septicaemia in a South Indian adult. J Infect Dev Ctries 6: 751–753.
14. Fantinati-Garbognin F, Almeida R, Portillo VA, Barbosa TA, Trevilato PB, et al. (2006) Drug resistance in Chromobacterium violaceum. Genet Mol Res 3: 134–147.
15. Szwarc LR, Szwarc DL, O’Loughlin CT, Giammanz R, Zhao B, et al. (2009) A quorum-sensing antagonist targets both membrane-bound and cytoplasmic receptors and controls bacterial pathogenicity. Mol Cell 35: 143–153.
16. Vasconcelos ATR, Almeida DE, Hungria M, Guimarães CT, Antonio RV, et al. (2003) The complete genome sequence of Chromobacterium violaceum reveals remarkable and exploitable bacterial adaptability. Proc Natl Acad Sci USA 100: 11650–11655.
17. Martindell D, Grossmann G, Seguin U, Brandl H, Bachofen R (2004) Effects of natural and chemically synthesized furanones on quorum sensing in Chromobacterium violaceum. BMC Microbiol 4: 25.
18. Kalia VC (2013) Quorum sensing inhibitors: An overview. Biotechnol Adv 31: 224–245.
19. Koh KH, Tham FY (2011) Screening of traditional Chinese medicinal plants for quorum-sensing inhibitors activity. J Microbiol Immunol Infect 44: 141–148. 
swarming motility and substrate analysis and O157: H7. Curr Microbiol 62: 1321–1330.

LuxI protein. Proc Natl Acad Sci biofilm formation and inhibition Vibrio harveyi extracts on the adhesion of early settlers in dental plaque to Chromobacterium

Escherichia Chromobacterium violaceum Chromobacterium over the quorum sensing behavior of is involved in the is essential for:ar eview wo of Yersinia pseudotuberculosis genome: molecular mechanisms associated with pathogenicity. Genet Mol Res 3: 148–161.

Bieber D, Ramer SW, Wu CY, Murray WJ, Tohe T, et al. (1998) Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic Escherichia coli. Science 280: 2114–2116.

Kennan RM, Dhungel OP, Whittington RJ, Egerton JR, Rood JJ (2001) The type IV fibrillar subunit gene (limA) of Dichelobacter nodosus is essential for virulence, protease secretion, and natural competence. J Bacteriol 183: 4433–4444.

Castro-Gomes T, Cardoso MS, DaRocha WD, Laiibda LA, Nascimento AM, et al. (2014) Identification of secreted virulence factors of Chromobacterium violaceum. J Microbiol 52: 330–333.

Miki T, Iguchi M, Akiba K, Hosono M, Sobue T, et al. (2010) Chromobacterium pathogenicity island 1 type III secretion system is a major virulence determinant for Chromobacterium violaceum-induced cell death in hepatocytes. Mol Microbiol 77: 855–872.

Atkinson S, Throup JP, Stewart GSAB, Williams P (1999) A hierarchical quorum-sensing system in Tersina pseudotuberculosis is involved in the regulation of motility and churning. Mol Microbiol 33: 1267–1277.

Yang Q, Defoirdt T (2014) Quorum sensing positively regulates flagellar motility in pathogenic V. harveyi. Env Microbiol doi:10.1111/1462-2920.12429 (in Press).

Lee J-HJ, Kim Y-GY, Cho MHM, Wood TKT, Lee JJ (2011) Transcriptomic analysis for genetic mechanisms of the factors related to biofilm formation in Chromobacterium violaceum. Curr Microbiol 57: 469–476.

Wood TK (2009) Insights on Escherichia coli biofilm formation and inhibition from whole-transcriptome profiling. Environ Microbiol 11: 1–15.

Lee JJH, Kim Y-GY, Cho MHM, Wood TKT, Lee JJ (2011) Transcriptomic analysis for genetic mechanisms of the factors related to biofilm formation in Escherichia coli O157: H7. Curr Microbiol 62: 1321–1330.

Liu Z, Wang W, Zhu Y, Gong Q, Yu W, et al. (2013) Antibiotics at subinhibitory concentrations impair the quorum sensing behavior of Chromobacterium violaceum. FEMS Microbiol Lett 341: 37–44.

Gu X, Xie Z, Wang Q, Liu G, Qu Y, et al. (2009) Transcriptome profiling analysis reveals multiple modulatory effects of Ginkgo biloba extract in the liver of rats on a high-fat diet. PLoS One 4: e6763.

Fathallah AR, Rahim ZHA (2003) The anti-adherence effect of Piper betle and Psidium guajava extracts on the adhesion of early settlers in dental plaque to saliva-coated glass surfaces. J Oral Sci 45: 201–206.

Deborde T, Boon N, Bossier P (2010) Can Bacteria Evolve Resistance to Quorum Sensing Disruption. PLoS Pathog 6: e1000989.