

Gelidiella acerosa extract as quorum sensing inhibitors of *Chromobacterium violaceum* and *Vibrio harvei*

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Abstract

Screening of marine macroalgae for quorum sensing inhibitors (QSIs) has gained great interest as they are the potential source of bioactive compounds and reports on QSIs from this source are scanty. Hence, the present study was to explore the QSI potential of the macroalgae to hinder quorum sensing (QS) mechanism of biomarker strains. Among the tested three seaweed extracts, methanolic extracts of *G. acerosa* was found to hinder QS dependent violacein pigment production in *Chromobacterium violaceum* (CV 12472) without showing any hindrance effect on bacterial growth. The *in vitro* quantification assays demonstrated that extract of *G. acerosa* repressed the violacein production up to 96.5% in *C. violaceum* CV026 and bioluminescence by 44.7% in *Vibrio harveyi* by interfering with their respective QS mechanism. Cell viabilities of test strains were not affected in the presence of *G. acerosa*. The results suggest that the macroalgae *G. acerosa* is an important source of QSI compounds. Consequently, identification and evaluation of active principle could be explored for prospective biotechnological applications.

Keywords: macroalgae, *Gelidiella acerosa*, quorum sensing, violacein, bioluminescence

Introduction

Quorum sensing (QS), also called cell to cell communication is an important phenomenon which determines the capability of pathogenic bacteria to detect and coordinate surrounding population to control genes responsible for pathogenicity (Fuqua and Greenberg, 2002). This communication system is regulated by tiny chemical molecules capable of diffusing termed autoinducers. N-acyl homoserine lactones and oligopeptides act as autoinducer signal molecules in Gram-negative and Gram-positive bacteria, respectively. Chemical signals secreted out by bacteria disseminate out and assemble in the surrounding environment. After reaching a threshold concentration, they bind with appropriate receptors and activate the transcription of target genes accountable for the violacein, a purple-coloured pigment synthesis (McLean *et al.*, 1997) [16] and bioluminescence emission (Nealson, 1977) [17].

A Gram-negative soilborne bacterium *Chromobacterium violaceum* is able to synthesize a violet-coloured pigment termed violacein through QS signal molecule HHL (N - hexanoyl homoserine lactone). The production of this signal molecule synthesized by the autoinducer synthase CviI will subsequently bind with its cognate receptor CviR to trigger the transcription of genes concerned with violacein production (Choo *et al.*, 2006) [5]. Autoinducer synthase CviI deficient strain of *C. violaceum* CV026 (Mc lean *et al.*, 1997) [16] which is a mutant, not able to synthesize violacein by its own, hence it necessitates an exogenous supply of HHL to carry out the pigment production. This bacterium is being considered as universal marker and is commonly employed to detect QS inhibitors.

Vibrio harveyi is the primary cause of the infectious disease called luminous vibriosis which accounts for the mortality of cultured shrimp in hatcheries globally (Liu and Lee, 1998). The pathogenesis of *V. harveyi* is established by the attachment of *Vibrio* to the shrimp surface (Austin and Zhang, 2006) [2] to form specialized structures called biofilm. Development of biofilms is regulated by signal mediated quorum sensing (QS) mechanism (Winzer and Williams, 2001) [21]. *Vibrio spp.* utilizes three distinct QS signaling molecules namely 3-hydroxy-C4-N-(3-hydroxybutanoyl)-l-homoserine lactone (AHL) otherwise called harveyi auto-inducer-1, HAI-1, 4,5-dihydroxy-2,3-pentanedione (DPD or Autoinducer-2, AI-2), and (S)-3-hydroxy tridecan-4-one (cholera autoinducer-1 or CAI-1). It is worth while noting that several virulence associated behaviors including bioluminescence in *Vibrio spp* are controlled by AI-2 based QS system (Winzer and Williams, 2001) [21] in which, AI-2 molecules synthesized by S-ribosyl-homocysteinase (LuxS) binds to LuxP, which directly linked with the protein sensor (LuxPQ) which encompass a conserved region of histidine kinase that coordinates the regulation of QS system. (Chen and Xie, 2011) [4]. As the emission of bioluminescence is regulated through AI-2 based QS (Henke and Bassler, 2004) [11], sighting compounds that hinder the AI-2 system could reduce bioluminescence and subsequent virulence.

Considering the importance of quorum sensing (QS) in bacterial pathogenesis, universal markers strains such as *C. violaceum* CV026 and *Vibrio harveyi* have been employed to screen QS inhibitors from various sources. Marine ecosystems still comprise of an unexploited resource for drug discovery. Interestingly, marine macroalgae

(seaweeds) are recognized to employ an excess quantity of metabolic by products to protect themselves from predator's attack and other microbial accumulation surrounding the uncovered areas. Hence, there is a rising interest in exploring the importance of marine macroalgae as a source of QS inhibitors. Several macroalgae have been found to attenuate bacterial QS signals (Givskov *et al.*, 1996)^[10]. The red alga *Delisea pulchra* produced halogenated furanones showed antibiofilm effects against various pathogens including *Escherichia coli* (Ren *et al.*, 2001)^[18], *Pseudomonas aeruginosa* (Hentzer *et al.*, 2002)^[12] and *Bacillus subtilis* (Ren *et al.*, 2002)^[19]. Also, a red marine macro alga *Asparagopsis taxiformis*, was reported to inhibit *C. violaceum* (Jha *et al.*, 2013). Similarly, solvent extracts macroalgae from the marine algae Arraial do Cabo, Brazil, exhibited QS inhibitory activity against *C. violaceum* CV017 (Batista *et al.*, 2014)^[3]. Recently, Carvalho *et al.* (2016)^[7] reported the QS inhibitory potential of the macro algae *Canistrocarpus cervicornis* against CV017. In this light, the present study was intended to investigate the QS inhibitory the activity of marine macroalgae against QS mediated violacein production in *C. violaceum* and bioluminescence in *V. harveyi*.

Materials and Methods

Bacterial Strains and growth conditions

The bacteria used for violacein inhibition studies such as *Chromobacterium violaceum* (ATCC 12472) and *C. violaceum* CV026 were grown in Luria-Bertani (LB) medium (pH 7.0 ± 0.2). For bioluminescence inhibition studies, *V. harveyi* (MTCC 3438) was employed and grown in marine Luria Bertani (mLB) broth (pH 7.5 ± 0.2) at 30°C. These three strains were maintained at 30°C. The strains were subcultured in LB until reach the cell concentration (1 × 10⁸ CFU ml⁻¹) for experimental analysis.

Extraction of Seaweeds

The seaweed samples such as *Gellidella acerosa*, *Turbinaria* spp. and *Gracillaria edulis* were extracted as described by Abraham *et al.* (2011)^[1]. The marine seaweed samples were dried and ground to fine powder. 5 g of powdered sample from each seaweed was marinated in methanol (50 mL) for 12 h. subsequently; the upper methanol phase was separated followed by evaporated. The dried pellets were resuspended in sterile water and kept at 20 °C for further experiments.

Violacein Inhibition Assay

Qualitative screening of QS inhibitors was carried out by using the wild biomarker strain *C. violaceum* (ATCC 12472). Sample that showed QSI activity was alone further subjected to quantification of violacein inhibition with the mutant strain CV026, a that is able to detect the synthesis of violacein production in when supplied with exogenous addition of N-hexanoyl-L-homoserine lactone (C6- HSL). For primary qualitative screening, microtitre plates (MTP) containing 1 mL of LB broth was added with 10 µL of wild strain *C. violaceum* 12472 (1 × 10⁸ CFU ml⁻¹) and grown without and with seaweed extracts at different

concentrations (100-500 µg/mL). The MTP was incubated for 16 h at 30 °C and monitored for violacein inhibition. Further, to quantify the amount of violacein produced in the presence of seaweed, the above-mentioned experiment was performed with the mutant strain *C. violaceum* CV026 along with addition of 5 mM C6-HSL. At the end of incubation, 1 mL of cell suspension from each well was taken up and subjected to centrifugation at 10,000 rpm for 10 min in order to pelletize the bacterial cells containing insoluble violacein. The remaining supernatant was removed. The cell pellet was redissolved in 1 mL of dimethyl sulfoxide to extort the violacein pigment. The mixture was then vortexed for 30 sec followed by centrifuged at 10,000 rpm to separate the extracted violacein from bacterial cells. The violcein was quantified using spectrophotometer (Hitachi U-2800, Japan) at 585 nm (Abraham *et al.*, 2011)^[1]

Inhibition of bioluminescence by *G. acerosa*

To measure the inhibition of bioluminescence, *V. harveyi* cells (1 × 10⁸ CFU ml⁻¹) were inoculated into glass tubes containing alkaline peptone water (APW) broth (5 MI) and grown without and with varying concentrations (100-500 µg/mL) of *G. acerosa*. Then the cells were allowed to grow for 16 h at 30 °C. Subsequently, bioluminescence produced by *V. harveyi* was recorded using luminometer as relative light units (RLU) (Teasdale *et al.*, 2009)^[20]

Inhibition percentage of bioluminescence = [RLUs of untreated Control – RLUs of Test / untreated control RLUs] × 100

Result and discussion

Violacein Inhibition by *G. acerosa*

Results of primary screening revealed that the extracts of *G. acerosa* exhibited a profound violacein inhibitory action against wild strain not interrupting the bacterial growth, whereas the extracts of *Gracillaria edulis* reduced the violacein production by affecting the bacterial growth. In contrast, *Turbinaria* spp. did not show any violacein inhibitory activity (Fig.1). Therefore, the extract of *G. acerosa* was alone subjected to quantitative analysis of violacein production using CV026. Results of quantitative analysis revealed a marked decrease in violacein content by not affecting the viable growth of *C. violaceum*. Results suggested utmost 96.47% violacein production got inhibited by the seaweed *G. acerosa* at (500 µg/mL) (Fig.2). The non-growth inhibitory effect of *G. acerosa* was confirmed by measuring the absorbance of CV 12472 and CV026 cultures at 600 nm supplemented with or without *G. acerosa* extract after 16 h of incubation. No considerable difference in growth was observed between untreated control and *G. acerosa* treated cultures (Fig.4) suggest that the observed reduction in violcein production was not due to quorum rather than sensing. Together, these results suggest the QSI potential of *G. acerosa*. The observed results obtained was found similar to the findings of Jha *et al.* (2013), wherein, the extracts of marine macro algae *Asparagopsis taxiformis* showed QS inhibitory activity against CV026 by reducing the production of violacein pigment.

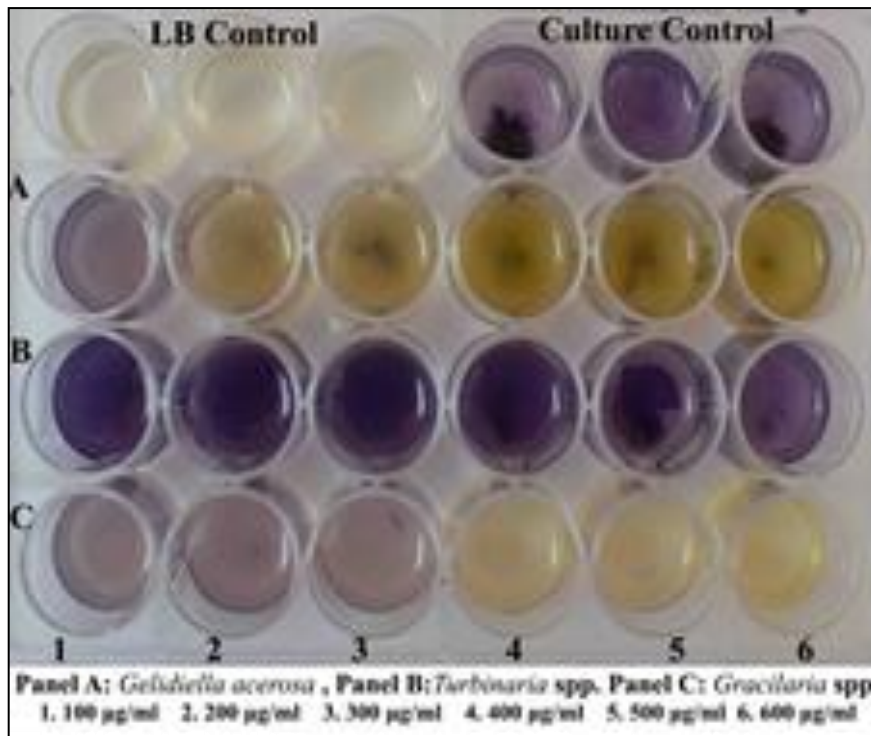


Fig 1: Qualitative analysis of Violacein inhibition against *C. violaceum* 12472 by marine seaweeds

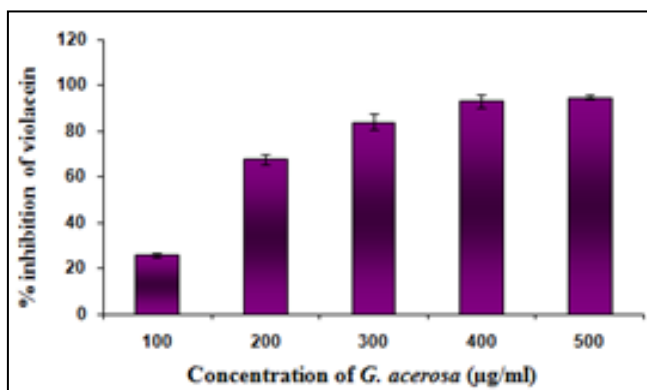


Fig 1: Quantitative analysis of violacein inhibition by *G. acerosa* against CV026

Bioluminescence inhibition assay

As the emission of *V. harveyi* bioluminescence is associated with signal mediated QS system, the QSI potential of *G. acerosa* was examined to impede the emission of bioluminescence by *V. harveyi*. The obtained results revealed that the *G. acerosa* inhibited the bioluminescence and the activity was depended on concentration. However, at higher concentration (500 µg/mL) *G. acerosa* inhibited the bioluminescence by 44.98 % (Fig.3) without showing any hindrance effect on bacterial growth. Prior to bioluminescence measurement the cell viability was examined by measuring the absorbance of test cultures spectrometrically at 600 nm. The attained results lucidly revealed that the number of feasible bacteria present in the extract treated was not different from the untreated control (Fig.4). These results were correlated with the decreased bioluminescence of *V. harveyi* upon treatment with *G. acerosa* (Fig. 3), suggesting that the significant inhibition in bioluminescence of *V. harveyi* treated with *G. acerosa* did not cause by the growth inhibition. The QS inhibitory potential of *G. acerosa* was similar to the QSI activity of *D. pulchura* derived furanones (Ren *et al.*, 2001) [18]. These

results were also fall in line with the very recent report by Karnjana *et al.* (2020), in which the bioactive compound N-benzyl cinnamamide derived from *Gracilaria fisheri* inhibited the QS dependent bioluminescence in *V. harveyi*.

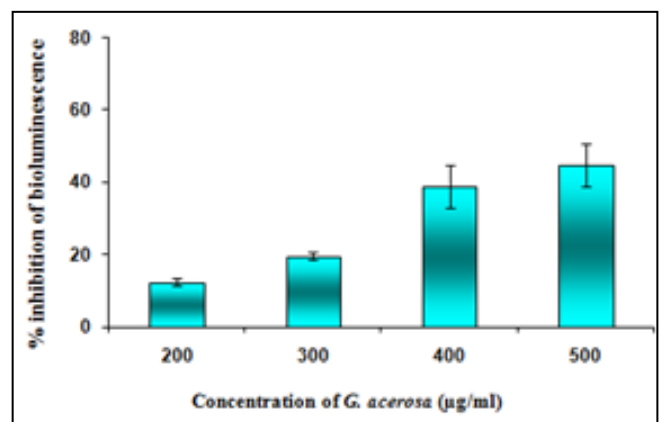


Fig 3: Inhibition of *V. harveyi* bioluminescence by *G. acerosa*

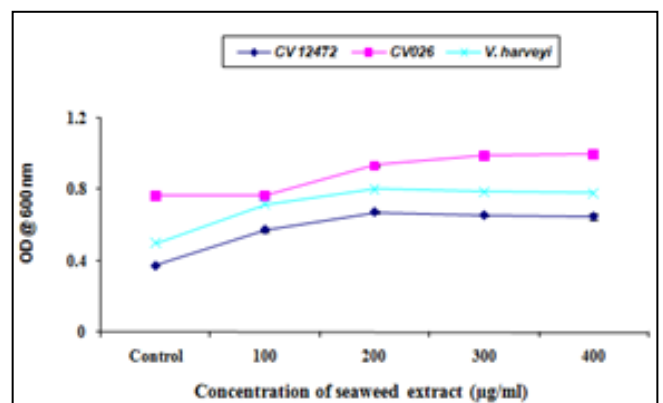


Fig 4: Effect of *G. acerosa* on growth of biomarkers

Conclusion

This study determined the QS inhibitory potential of marine seaweed *G. acerosa*. Violacein and bioluminescence assays revealed that *G. acerosa* interfered with QS mechanism of biomarker strains. Hence, isolation and purification of lead compounds from *G. acerosa* will provide a new insight on the development of QS inhibitors from marine microalgae which potentially can be used in drug development.

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Conflict of Interest

The authors have no conflict of interests

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