Quorum sensing inimical activity of *Tribulus terrestris* against gram negative bacterial pathogens by signalling interference

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Abstract

Bacterial virulence is controlled by a cascade of genes influenced by quorum sensing alias bacterial signalling. The present study was intended to develop an effective module that could constrain bacterial communication without harming the host. Quorum quenching ability of *Tribulus terrestris* was screened upon chromogenic reporter strains such as *Chromobacterium violaceum*, *Serratia marcescens* and *Pseudomonas aeruginosa*. Hydro-alcoholic extracts of root showed positive quorum quenching activity by effectively down regulating quorum sensing controlled mechanisms such as pigment production and biofilm formation. Lead component was purified and found to be β -1, 5-O-dibenzoyl ribofuranose by GC–MS NIST and NMR spectrometry. Interestingly it was observed that the compound was neither bactericidal nor bacteriostatic but rather it's only disturbing its interaction. Further studies revealed that the antagonist is not inhibiting the production of signalling molecule acyl homoserine lactone, instead inhibiting its action.

Keywords Acyl homoserine lactone · β -1 · 5-O-dibenzoyl ribofuranose · Quorum sensing · Quorum quenching · *Tribulus terrestris*

Introduction

Ever since the origin of life in earth, the microorganisms are in constant interaction with both biological entities and non-living things. Several strategies have been employed to annihilate microbial pathogens which are collectively called as antibacterial therapies. Antibiotic drugs were found to be most prominent among all antibacterial therapies. Antibiotics targets essential processes for bacterial existence such as cell wall synthesis, DNA replication, transcription and protein synthesis (Kohanski et al. 2010).

Even after an impeccable track record, the efficacy of antibiotic therapy have been questioned ever since the discovery of first antibiotic resistant *Staphylococcus aureus* in mid-nineteenth century, since then many antibiotic resistant strains such as *E. coli* O104, *K. pneumoniae* NDM-1 were delineated. Currently over 70% of total pathogens have been

acclaimed for their resistance towards at least one of the commercially available antibiotics (Hong et al. 2012). To make the scenario worst, discovery of new antibiotics has become scarce resulting in an urge to implement alternative therapeutic methods to combat pathogens by targeting their virulence.

According to the current scenario, studies on quorum sensing (QS) concept and compounds with quorum quenching capability have gained a vital impact. Quorum sensing is a density dependent mechanism that allows the bacterial community to initiate secondary functions mostly related to virulence under a threshold population mediated by small signalling molecules autoinducers (AI). Bacterial functions such as pigment production, biofilm formation, swarming motility, toxin production and exozyme production are found to be controlled by quorum sensing (Waters and Bassler 2005). Based on its mechanism three quorum sensing systems have been documented such as acyl homoserine lactone (AHL) mediated quorum sensing which is exclusively seen in gramme negative bacteria, LuxS controlled autoinducer-2 mediated communication seen in both gramme negative and gramme positive bacteria, finally peptide induced signalling which is spotted in gramme positive bacteria (Rutherford and Bassler 2012).

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Inhibition of quorum sensing is called as quorum quenching where the signalling mechanism is inhibited without disturbing bacterial growth. As a consequence, host defence mechanism gets adequate exposure for effective immune clearance (Chen et al. 2013). In other way quorum quenching can also be explained as in vivo attenuation of pathogens. Quorum quenching is achieved by either degrading autoinducers or by blocking quorum sensing signals. Halogenated furanone extracted from red algae *Delisea pulchra*. Stands the first report of quorum quenching natural product. Since then many natural products have been screened for its efficacy to antagonise quorum sensing signalling (Tang and Zhang 2014). Even after an immense understanding about its vital importance, till date only a very limited number of quorum quenching modules have been successfully adopted to the clinical level due to their low biocompatibility. This insists the research society to find a nontoxic remedial module from pre-existing medical traditions against quorum sensing mediated virulence. Studies carried out upon some traditional Hispanic (Huerta et al. 2008), Chinese (Yeo and Tham 2012), Egyptian (Aki et al. 2013) and Mongolian (Zolboo et al. 2014) medicines possessed promising quorum quenching.

In this study the quorum sensing inimical activity of *Tribulus terrestris* was analysed and this stands the first ever report of quorum quenching activity by the same.

Materials and methods

Sample collection and bioactive compound isolation

Herbal plant *Tribulus terrestris* was collected from Western Ghats, Kerala, India. Roots were taken and washed in sterile water which was then dried and powered. Extracts were taken in low-polar, mid polar and polar solvents by the employment of hexane, 80% methanol and water respectively by addition of solvents into sample and subjecting the mixture for agitation at room temperature for 48 h. After incubation extracts were filtered through Whatman No.1 philtre paper and later concentrated in vacuum (Keshtmand et al. 2015). Fraction that with positive quorum quenching activity was taken into further studies.

Bacterial strains and maintenance

The activity of the quorum quenching compound was screened by using chromogenic bacterial strains. Type strains *Chromobacterium violaceum* MCC 2290 (wild strain) and *Chromobacterium violaceum* CV26 MCC 2216 (mutated non-chromogenic strain) were obtained from NCCS, Pune,

India. Chromogenic bacterial strains *Pseudomonas aeruginosa* strain SV1 (NCBI accession number KT259196) and *Serratia marcescens* strain BJV5 (NCBI accession number KM018333) were isolated from digestive system of marine squid. All the strains were maintained in Luria–Bertani (LB) agar slants in 4 °C.

Analysis of quorum quenching potential

Overnight broth culture of *Chromobacterium violaceum* CV26 was inoculated onto molten Luria–Bertani (LB) agar plates supplemented with *N*-3-oxohexanoyl-homoserine lactone (0.25 mg/ml). Root extract incorporated onto a sterile disc (2 mg/disc) was placed over bacterial lawn culture. Disc loaded with solvent and with distilled water was maintained as control. Optimum concentration required for quorum quenching action was determined by administering the extract in varying concentrations (0–3 mg/ml) in above mentioned liquid bacterial culture (Zhu et al. 2011).

Activity dependent purification and identification of bioactive compound

Crude methanolic extract possessing positive quorum quenching activity was subjected for silica gel column chromatography (60–120 mesh) and the bioactive compounds were eluted by the employment of appropriate solvents of varying polarity. Eluted fractions were screened for their quorum quenching activity; finally, positive fraction was concentrated and the lead component was identified by the employment of GC–MS, NIST library and confirmed by the employment of NMR spectrometry (Jothy et al. 2011).

Effect of compound in signal synthesis and activity

Effect of compound in synthesis and activity of AHL was determined by employment of reporter strains. *Chromobacterium violaceum* wild strain and *Chromobacterium violaceum* CV26 were grown in the presence of increasing concentrations of the β -1, 5-O-dibenzoyl ribofuranose. Strain CV26 culture was supplemented with *N*-3-oxohexanoyl-homoserine lactone as a signalling molecule. After overnight incubation, AHLs from *Chromobacterium violaceum* wild strain culture was extracted (Bryant et al. 2008) and added to a new liquid culture of *Chromobacterium violaceum* CV26 in the absence and presence of antagonist to check its activity (Vasavi et al. 2013).

Quorum sensing inhibition in wild isolates

Capability of root extract to modulate quorum sensing in other wild isolates such as *Pseudomonas aeruginosa* strain

SV1 and *Serratia marcescens* strain BJV5 were investigated. Strains were grown in LB broth media inoculated with different concentrations of antagonist (0–2.5 mg/ml). Parameters such as growth, pigment production (Chu et al. 2013) and biofilm formation (Burt et al. 2014) were determined.

Fig. 1 a Quorum quenching mediated non pigmentation zone in different concentrations of antagonist. b Quorum sensing dependent pigment production is seen reduced with respect to increased concentration

Results

Screening and quantification of quorum quenching activity

Extracts of *Tribulus terrestris* showed positive quorum quenching properties which was evident by the formation of non-pigmented turbid zone of violacein inhibition at the site of administration (Fig. 1a). Activity of extract was found to be directly proportional to its concentration, as higher activity was evident with higher concentration. Maximum activity was found in the concentration of 2.5mg/ml however concentration less than 0.5mg/ml did not show any quorum quenching activity (Fig. 1b). Interestingly the bacterial biomass remained unaffected by quorum quenching antagonist.

Characterization of bioactive compound

Activity dependent purification of chromatographic fractions aided the successful retrieval of bioactive compound. Column chromatography of *Tribulus terrestris* extract gave 30 fractions on eluting with hexane: petroleum ether (1–7 fractions), petroleum ether: ethyl acetate (8–15 fractions) and last 15 fractions eluted by methanol. Compounds were grouped according to the R_f values. The methanol fraction gave single spot indicating single compound. Fractions that with positive quorum quenching action were concentrated and recrystallized later analysed by GC–MS NIST. Lead compound was identified as β -1, 5-O-dibenzoyl ribofuranose at the retention time of 8.97 min constituting of two benzene ring attached to a central penta ring (Fig. 2). Further the compound was verified by NMR spectrum (Fig. 3) through which the molecular structure of compound elucidated

Fig. 2 a Chromatogram of purified bioactive compound. b Mass spectrometry data. c Structure of bioactive compound

Fig. 3 a ^1H -NMR spectrum, b ^{13}C -NMR spectrum

from the spectrum for which the following chemical shift was following ^1H NMR: (500 MHz, CDCl_3) δ 3.1 (1H, dd, $J=7.1$, 5.4 Hz), 3.7 (1H, dd, $J=7.1$, 4.4 Hz), 7.41–7.51 (4H, $J=8.5$, 7.5, 1.3, 0.4 Hz), 7.56–7.63 (2H, tt, $J=7.5$, 1.5 Hz), 7.99–8.07 (4H, m, $J=8.5$, 1.9, 1.5, Hz). ^{13}C NMR (125 MHz, CDCl_3): δ 128.2, 129.1, 130.6, 49.2, 40.2, 39.9, 2.1.

Effect of antagonist in signal production and signalling

AHL dependent violacein production in *Chromobacterium violaceum* wild strain was found to be reduced in the presence of antagonist. Interestingly AHL molecules extracted from the above culture could initiate pigment production in external AHL dependent mutated strain *Chromobacterium violaceum* CV26. This suggests that plant extract have no role in inhibiting AHL production rather it disrupt the interaction between signalling molecules and response gene. This was proved as the *Chromobacterium violaceum* CV26 strain could not possess chromogenesis in the presence of compound even after the addition of AHL into the growth media (Fig. 4).

Quorum sensing hostility in wild isolates

Antagonistic activity of β -1, 5-O-dibenzoyl ribofuranose against the isolates SV1 and BJV5 was promising, moreover results justified the observations made in type strain. Biofilm formation and pigment production were found reducing whereas the growth remained undisturbed. About 94% of reduction in pigment production was observed in SV1 and biofilm formation dropped about 91% in the presence of 2.5 mg/ml concentration of antagonist. Quorum sensing

Fig. 4 AHL modulation mechanism by extract. A Wild strain in the presence of antagonist, B mutated strain CV 26 supplemented with AHLs extracted from A, C mutated strain CV26 in the presence of AHLs and antagonist

controlled chromogenesis and cell adherence was found to be diminished in strain BJV5 by 90% and 93% respectively (Fig. 5a, b).

Discussion

Natural products are most desired quorum sensing antagonists because of its dominance over synthetic counterparts with high biocompatibility. *Tribulus terrestris* is a popular medicinal plant that has been widely used in ancient Chinese as well as Indian medicinal system. Plant is used as an aphrodisiac, analgesic, astringent, anti-hypertensive and diuretic activity (Soleimanpour et al. 2015). Quorum sensing antagonistic action of root could be the one possible reason behind its inclusion among *dasamoolam* (dasam: ten, *moolam*: root) which is a combination ten roots based

Fig. 5 **a** Quorum quenching activity of compound against isolate *Pseudomonas aeruginosa* SV1. **b** Quorum quenching activity of compound against isolate *Serratia marcescens* BJV5

on Ayurveda system. So far many such quorum quenching plants were reported such as vanilla (Nur et al. 2013) and coffee plant (Choo et al. 2006).

Further studies conducted on signalling in *Chromobacterium violaceum* CV26 gave a clear picture about antagonistic action of β -1, 5-O-dibenzoyl ribofuranose. In the presence of antagonist, wild strain failed chromogenesis which is supposed to be a natural phenotype of wild strain controlled by quorum sensing. But AHLs extracted from the above mentioned non chromogenic wild strain could initiate pigment production in mutated strain *Chromobacterium violaceum* CV26 which demand external addition of AHLs. Similarly leaf extracts of *Syzygium cumini* and *Pimenta dioica* exhibited quorum quenching upon reporter strain *Chromobacterium violaceum* CV26 (Vasavi et al. 2013). These observations suggests that the compound lack the capacity to inhibit the production of AHLs instead it blocks the interaction between signalling molecule and response gene which emphasise the possibility of competitive inhibition of signalling molecule by the compound.

Quorum quenching without disturbing the growth of organisms was found to be successful against isolates *Pseudomonas aeruginosa* SV1 and *Serratia marcescens* BJV5 which highlights its versatility against vast array of pathogens similar to that of Yayurea A and Yayurea B purified from *Staphylococcus delphini* (Chu et al. 2013). Out of the three parameters analysed (growth, chromogenesis and biofilm formation), growth of organism was found to be unaffected by signalling antagonist whereas pigment production

and biofilm formation reduced in a proportion with increasing concentration of extract. A similar result was reported where malabaricone C from *Myristica cinnamomea* successfully reduced virulence in quorum sensing *Pseudomonas aeruginosa* strain PAO1 (Chong et al. 2011). In immunological aspect, incapability of a pathogen to develop biofilm prolongs its latent phase inside a host which presents an opportunity to host defence mechanism for successful immunological clearance.

Earlier in vivo action of quorum quenching compounds have been found successful against *Pseudomonas aeruginosa*. Furanone C30, a synthetic derivative significantly affected transcription of *lasRI* and *rlhRI* genes (Hentzer et al. 2003) and enhanced immunological clearance. Garlic extract exhibited effective signal silencing in Urinary tract infection (Harjai et al. 2009) as well as lung infection in mice (Bjarnsholt et al. 2005). In contradiction to current antibiotic therapy quorum quenching drugs are not taking the role of host immune system but rather only supporting it for complete persisting pathogenic neutralisation.

Conclusions

Root of *Tribulus terrestris* was found an effective quorum quenching agent. It didn't influence the growth of organism whereas distracted signalling communication of gramme negative bacteria. This property can be further exploited for in vivo attenuation of gramme negative pathogens.

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Compliance with ethical standards

Conflict of interest We declare no conflict of interest.

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