Strategies for impeding quorum sensing in bacteria: Basic and applied aspects

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In
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CERTIFICATE

This is to certify that the work incorporated in this Ph.D. thesis entitled **"Strategies for impeding quorum sensing in bacteria: Basic and applied aspects"** submitted by **Ms. Amrita Patil** to Academy of Scientific and Innovative Research **(AcSIR)** in fulfillment of the requirements for the award of the Degree of **Doctor of Philosophy in Biological sciences** embodies original research work under our supervision at the Biochemical Sciences Division, National Chemical Laboratory, Pune- 411 008, India. We further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources, have been duly cited and acknowledged.

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I hereby declare that the thesis entitled **"Strategies for impeding quorum sensing in bacteria: Basic and applied aspects"** is completely genuine and the work towards this thesis was carried out by me under the kind supervision of Dr. Dhanasekaran Shanmugam (Research supervisor) and Dr. Asmita Prabhune (Co-supervisor) at the Division of Biochemical Sciences, CSIR- National Chemical Laboratory (NCL), Pune, India. The thesis is being submitted by me to AcSIR for the degree of Doctor of Philosophy in Biological sciences.

I further affirm that the subject matter of this thesis has not formed the basis for award of any other degree, diploma, associate-ship, and fellowship, in this or any other University or other institution of higher learning. The material obtained from other sources has been duly acknowledged throughout the thesis.

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Amrita Patil

This thesis work is dedicated to

my Parents

and

all the researchers who have contributed to the field of quorum sensing

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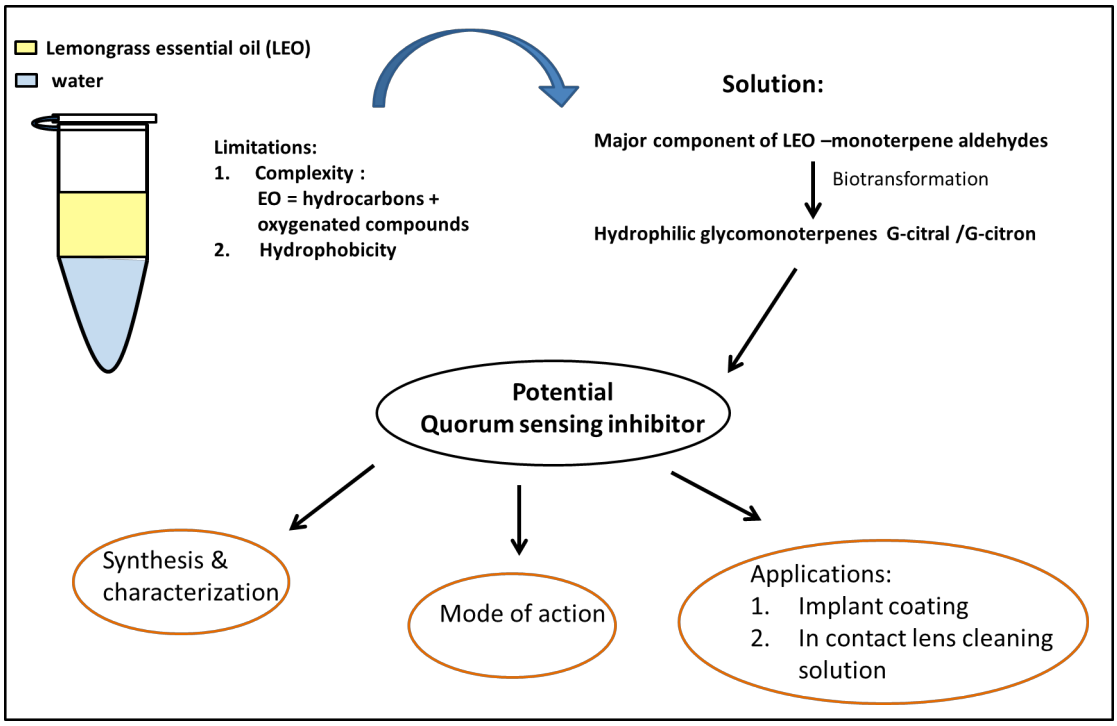
Introduction

Strategies for impeding quorum sensing in bacteria: Basic and applied aspects



1.1 History of Quorum sensing

Quorum sensing (QS) is a phenomenon in which one bacterium sense other bacteria in the surrounding due to the accumulation of signaling molecule produced by them. The discovery of quorum sensing invalidated our earlier assumption of the singular existence of bacteria. Before 1994, quorum sensing was called as "autoinduction," and it was initially found in the Gram-negative marine bacterium *Vibrio fischeri* in the late 1960[1]. Kempner and Hanson in 1968 revealed kinetics of light emission by *V. fischeri* and also found out that transient depression in light emission can be excluded by conditioning medium by pre-exposing the medium to the same bacteria[2]. Later, it was discovered that the regulation of the synthesis of

bacterial luciferase occurs on the transcription level[3] and the process is controlled by extracellularly secreted AHL autoinducers (signal molecules) *N*-(3-oxohexanoyl)-homoserine lactone (3-oxo-C6-HSL)[4,5]. Hastings and his student Nealson (in 1970) found that, during the lag phase, luciferase gene (or operon) is repressed and in the exponential period of growth the luciferase gene is activated, and luciferase is rapidly synthesized. They named this phenomenon as "autoinduction"[3]. This phenomenon was studied further by many groups and in 1994 Dr. Steven Winans coined the term "Quorum sensing" for the same phenomenon in the review titled "Quorum Sensing in Bacteria: the LuxR-LuxI Family of Cell Density-Responsive Transcriptional Regulators" Fuqua et al. (1994)[6]. Previously it was believed that AHL-based QS system was only present in marine bacteria such as *V. fischeri* and *V. harveyi*. However, subsequent discoveries in this field revealed that QS is far more widespread than considered previously.

1.2 Quorum sensing systems:

Each bacterium uses different signal molecules for communication which are unique to them and do intra-species as well as interspecies communication. Based on signal molecule used by bacteria QS systems can be distinguished as follows.

1.2.1 Autoinducer type 1 system (AI-1):

This QS system is present in Gram-negative bacteria and driven by AI-1 i.e., AHL signal molecule.

Major elements and regulatory system of AHL quorum sensing system:

1. Signal molecule:

In this system bacteria synthesizes and use Acyl Homoserine Lactone (AHL) as the quorum sensing signal molecule. The AHL molecule contains a homoserine lactone (HSL) ring and the acyl chain with varying number of carbon atoms (4-18), the number of oxygen substitution and the degree of saturation (Figure1.1). AHLs with small acyl chain length and low molecular weight are freely diffusible molecules, however, AHLs having long acyl chain length and high molecular weight requires efflux pump for their transport through the membrane to detect bacterial densities in the surrounding[7,8].

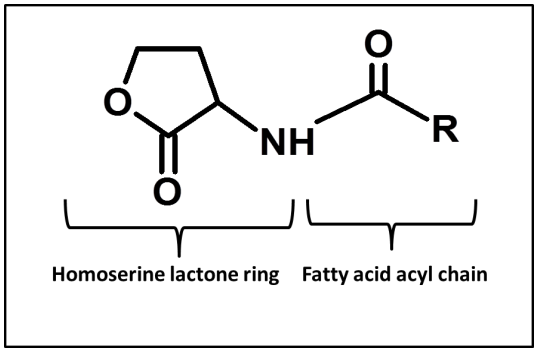


Figure 1.1: Structure of N-acyl homoserine lactone: The AHL molecule contains a homoserine lactone (HSL) ring which is bound to the acyl chain with varying number of carbon atoms.

2. Signal synthase:

AHL synthase enzyme is encoded by the *luxI* gene and is used for the synthesis of signal molecule in *V. fischeri*. Some other quorum sensing bacteria e.g. *Burkholderia, Agrobacterium*, *Rhizobium*, *Serratia*, *Pseudomonas,* and *Erwinia*, etc also have AHL synthase gene similar to *luxI*. *Pseudomonas aeruginosa* possess two types of AHL synthase LasI and RhlI producing 3-oxo-C12-HSL and C4-HSL respectively. LuxI homologs possesses some conserved residues at their active site so their mode of action is almost similar to each other[9]. AHL synthase produces AHLs from two substrate acylated acyl carrier protein (acyl-ACP) and S-adenosyl-L-methionine (SAM). In this reaction acyl carrier protein presents acyl chain to the AHL synthase resulting in acylation reaction through nucleophilic attack by amine group of SAM on the carbonyl carbon of acyl-ACP. Moreover, homoserine lactone ring is formed by lactonization reaction though nucleophilic attack on the γ carbon of SAM by its own carboxylate oxygen (Figure 1.2)[10]. Each AHL synthase has specificity to a particular chain length so one bacterial strain may possess multiple synthases to produce a variety of AHLs[9].

This AHL synthesis mechanism has two unique features. First, both the substrates acyl-ACP and SAM adopt different cellular functions than their usual role in fatty acid biosynthesis and as methyl donor respectively and second, during internal lactonization of SAM unique cyclic confirmation is attained to favor the reaction[10].

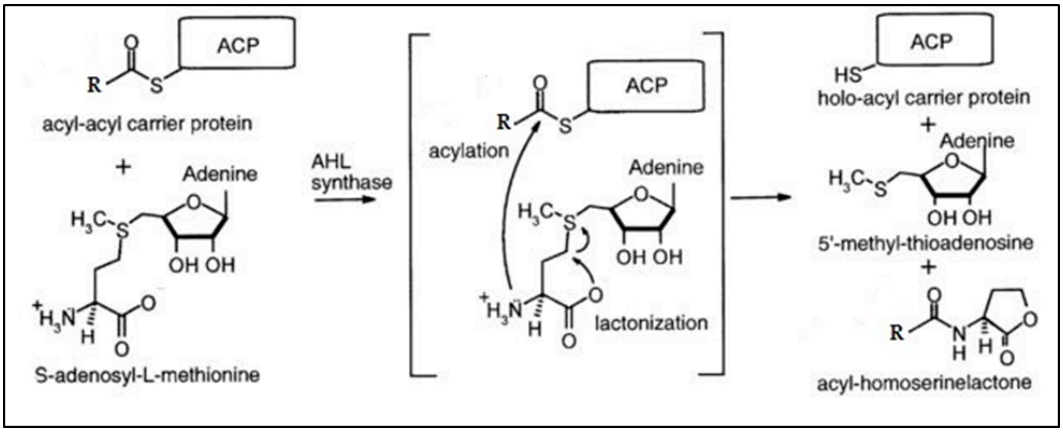


Figure 1.2: The schematic representation illustrating the reaction of AHL synthesis: SAM and acyl-ACP bind to the AHL synthase leading to acylation, and subsequent lactonization reactions through nucleophilic attack on the carbonyl carbon of acyl ACP and γ carbon of SAM by amine group and carboxylate oxygen of SAM respectively. AHL and byproducts holo-ACP and 5'-methylthioadenosine are formed during the reaction.

The second category of AHL synthases which are non-homologous to LuxI is LuxM and AinS which were first found in the *V. harveyi* and V. *fischeri* respectively. However, when protein was purified and studied further, it was observed that the synthesis mechanism is similar to LuxI with a unique aspect that acyl-coenzyme A (acyl-CoA) can also act as acyl donor like acyl-ACP. The third category of AHL synthase-HdtS, was discovered in *P. fluorescens*. This synthase is not similar to LuxI, LuxM or AinS-type synthases[9].

3. Signal receptor:

In the quorum sensing system, third essential element is signal receptor protein which are stimulated by binding to an auto-inducer molecule. Bacteria may possess multiple transcriptional regulator proteins depending upon the number of signal molecules it produce. *V. fischeri* possess LuxR protein which is activated upon binding to 3-oxo-C6-HSL. LuxR possess N terminal and C terminal domains for binding of ligand and DNA respectively. Specific amino acid residues at N-binding domain of LuxR interact with 3-oxo-C6-HSL. However, helix-turn-helix motifs at C-terminal domain interact with DNA at specific site consisting of 20bp inverted repeat upstream of transcription start site called as "lux box."

The earlier model describing the activity of LuxR type was established on the hypothesis that the N-terminal domain of these regulator proteins is associated with

the cytoplasmic membrane and upon binding to their specific ligand (AHL molecule), they undergo structural changes and form specific conformation which helps in binding to its target DNA. However, it was not known if the interaction of these proteins with the membrane is reserved after binding with AHL and with subsequent structural change. However, some recent studies corroborated earlier assumption of activity of LuxR type proteins and also revealed that these proteins are membrane-associated monomers which upon binding to AHL molecule dimerize and release into the cytoplasm in the soluble form and in the absence of their specific AHL molecule they are quickly destroyed by endogenous proteases after misfolding. (Figure 1.3)[11–15]. One live cell labeling study carried out by Rayo et al. (2011) also answered the another question about location of LasR (LuxR type protein). They found that LasR is present at the poles of the *P. aeruginosa* cell and not present equally all over the cytoplasmic membrane. This result indicated that there is compartmentalization in secretion and recognition of AHL molecules. Newly synthesized AHL molecules are secreted from the *P. aeruginosa* to the surrounding through the MexAB-OprM efflux pump and as per recent studies homologous efflux pump Opr86 is present at the middle part of the cell however as per Rayo et al. (2011), recognition of AHL occurs at poles of the cell[13].

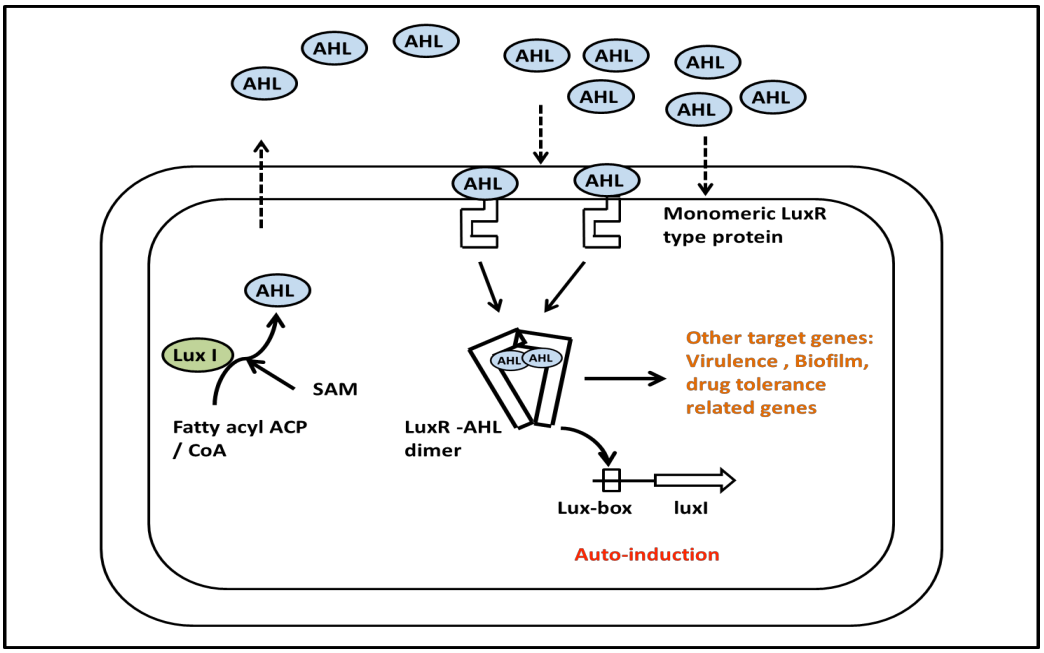


Figure 1.3: Autoinducer type 1(AI-1) or AHL based quorum sensing system in Gram-negative bacteria: Bacteria produce AHL auto-inducers with a varying number of carbon atoms (4-18), the number of oxygen substitution and the degree of saturation. AHLs are synthesized by enzyme LuxI with two substrates, acyl chain of fatty acid presented by either acyl carrier protein or CoA and SAM. Attainment of the threshold concentration of AHL outside the cell leads to recognition of AHL by N-binding domain of LuxR associated with the cytoplasmic membrane. Upon activation by AHL, LuxR dissociates from the membrane and change its conformation to form a dimer. C-terminal domain of AHL-LuxR dimer binds to Lux-box and autoinduces QS system by transcription of luxI gene. AHL-LuxR dimer also initiates expression of genes associated with QS mediated phenotypes (AHL: N-Acyl Homoserine Lactone; LuxI: all LuxI type signal synthase; CoA: Co-enzyme A; SAM: S-adenosyl methionine; LuxR: all LuxR type transcription regulator, Lux-box: LuxR-AHL dimer binding domain (conserved inverted repeats))

1.2.2 Autoinducer type- 2 system (AI-2):

This was first found in marine bacteria *V. harveyi*. Later, this QS was observed in Gram-negative as well as Gram-positive bacteria. Therefore, it is also called as universal QS system.AI-2 signal is produced by substrate S-4, 5-dihydroxy-2, 3-pentanedione (DPD) through S-ribosyl homocysteinase encoded by *luxS* gene. AI-2 signal molecule is composed of cyclical furanosyl molecules with or without a boron atom. For example, in *Salmonella* sp., R-THMF [(2R, 4S)-2-methyl-2,3,3,4-tetra-

hydroxy tetrahydrofuran], a molecule without boron act as AI-2. However, *V. harveyi* possess S-THMF-borate [(2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran - borate] as autoinducer type- 2 signal[7]. Receptor system in AI-2 system consists of two components e.g., *Vibrio* sp. receptor is bound to membrane and has kinase phosphatase active LuxQ and signal binding LuxP domains. However, in some enteric bacteria, the receptor is a soluble protein which recognizes signal at periplasmic space and signal is transported inside the cell through ABC transporter. Inside the cell, phosphorylation of signal molecule and further binding to an intra-cellular transcriptional activator occurs. As AI-2 signal synthesized by one bacterial species is recognized by another bacterial species it is also called as interspecies communication system[7].

1.2.3 Autoinducer type-3 system (AI-3):

AI-3 system is found in various commensal bacteria like enterohemorrhagic *E. coli* (EHEC), pathogenic *Shiegella*, *Salmonella,* and *Klebsiella sp*. AI-3 QS system consists of receptor kinase having two components. A signaling molecule of this system is unknown, but as per some studies structure is found to be similar to catecholamines. In case of enterohemorrhagic *E. coli* (EHEC), QseA controls a pathogenicity island LEE- the locus of enterocyte effacement and QseBC, associated with periplasmic space, recognizes AI-3 signal as well as human stress hormones epinephrine, norepinephrine and regulates the motility genes[16]. In QsceBC complex QseC functions as sensor kinase where as QseB act as response regulator with phosphorylation. As this system responds to human stress hormone, this system is also called as inter-kingdom system[7].

1.2.4 Quorum sensing system in Gram-positive bacteria:

This system involves small auto-inducing peptides (AIP) act as signal molecules. This system is also called as two-component system as it involves two proteins to initiate transcription of target genes.

In this system, oligopeptides are cleaved into small functional units and secreted out of the bacteria through ABC transporter protein. Once concentration of these signal molecules outside the cell attains a threshold concentration, gets detected by sensor kinase. Receptor protein gets autophosphorylate at histidine, and phosphate group shifts to response regulator protein at aspartate residue. Transcription of the desired

genes is led by the phosphorylated regulator protein bound to a specific DNA site (Figure 1.4) [17].

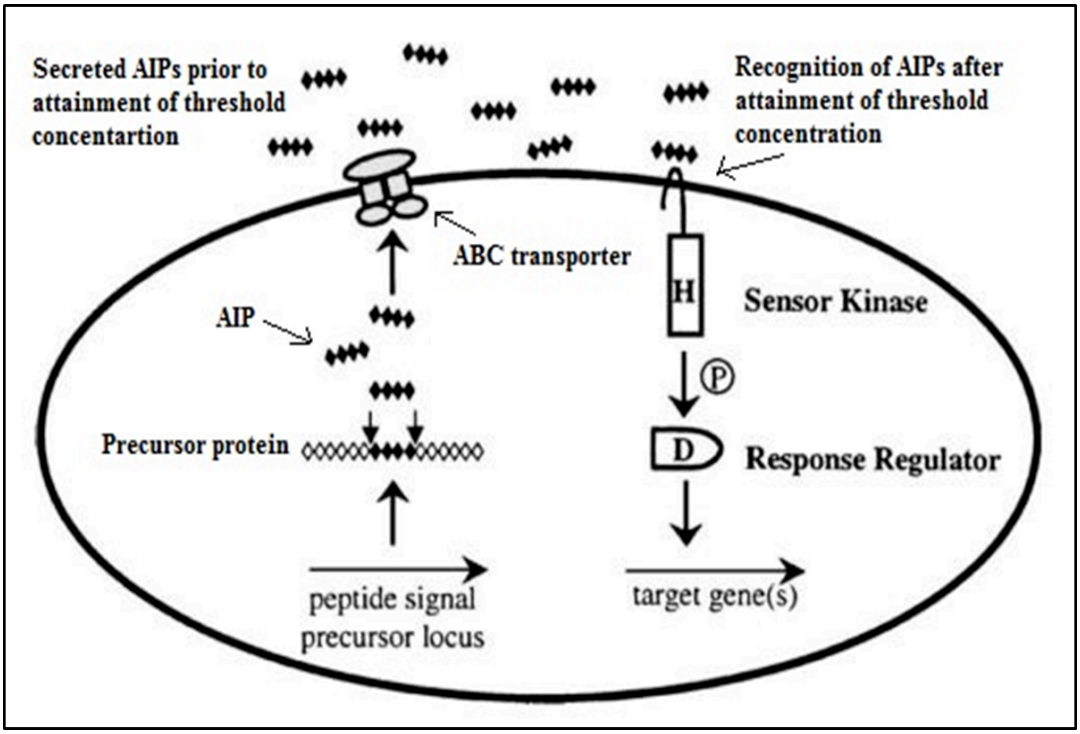


Figure 1.4: QS system present in Gram-positive bacteria: A precursor protein is synthesized and further split to form AIP signal. The peptide signal is commonly translocated to the surrounding through an ABC transporter. After attainment of the stimulatory threshold concentration of AIPs in the surrounding they are detected by two-component system wherein first it is autophosphorylated at histidine group (H). Further, the phosphate group of histidine kinase is shifted to aspartate (D) of response regulator. This subsequently initiates the transcription of desired genes (the figure is adapted from reference [17])

1.2.5 Other QS systems:

Apart from above mentioned major QS systems, many organisms also possess different QS systems which act in individually or in sequence and regulate expression of respective QS genes e.g. In addition to 2 major AHL quorum sensing systems (LasI/R, RhlI/R) *P. aeruginosa* also possess two other quorum sensing systems based on signals IQS (Integrated Quorum sensing Signal) and PQS (*Pseudomonas* quinolone signals). These two systems are mainly activated by LasI/R quorum sensing

system[18]. However, *V. cholerae* possess two QS systems the Cholerae autoinducer-1 (CAI-1/ (s)-3-hydroxytridecan-4-one) and AI-2 system[7].

1.3 Quorum sensing mediated phenotypes:

Bacterial communities regulate various collective functions using a phenomenon of quorum sensing. Quorum sensing systems enable bacteria to estimate the cell density of same or other species and also species from other kingdoms in the surrounding population and direct gene expression accordingly[19]. Major quorum sensing mediated phenotypes contributing for severe pathogenesis are mentioned in Figure 1.5.

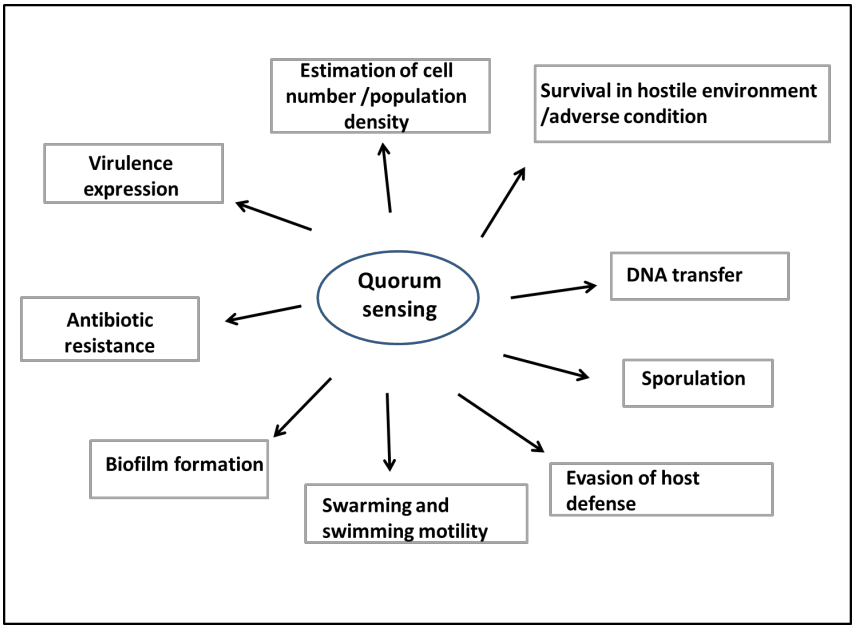


Figure 1.5: Quorum sensing mediated phenotypes (Figure adapted from reference[7])

1.4 QS and Biofilm formation:

1.4.1 Biofilm development:

Biofilm formation is one of the significant QS mediated phenotypes. Biofilms are sessile groups of microbes where bacteria are surrounded by self-produced extracellular matrix (ECM). Depending on the bacteria within the biofilm, the composition of the ECM varies, but usually, it consists of bacterial-derived components (complex polysaccharides, proteins, lipids, nucleic acids) and host factors (fibrin, platelets and immunoglobulins) (Figure 1.6).

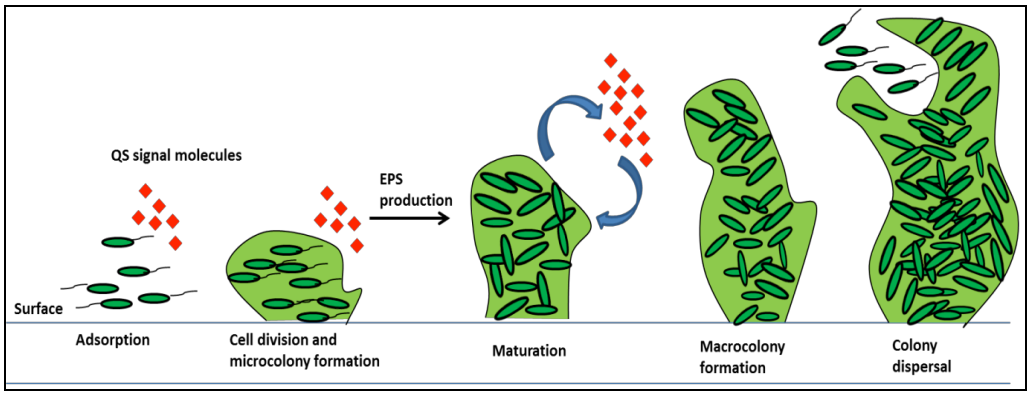


Figure 1.6: Steps involved in the *Pseudomonas aeruginosa* biofilm development.

1.4.2 Influence of QS on biofilm formation:

The role of QS in the biofilm formation was first explained by *Serratia liquifaciens* by Eberl et al. in 1996[20]. Later on, Davies et al. (1998) discovered that *P. aeruginosa* needs an active LasI/R QS system to develop mature biofilm and also showed that biofilm formed in the absence of LasI/R QS was more sensitive to SDS than in the presence of LasI/R QS system[21]. Similarly, QS stimulate biofilm development in numerous ways in various species. Some representative examples are mentioned in Table 1.1 (Table adapted from reference [22])

Table 1.1: Influence of the QS in biofilm development

Organism	**Inactivated QS system**	**Influence on biofilm development**
Aeromonas hydrophila	ahy-AHL	Affected maturation of biofilm
Burkholderia cepacia	cep- AHL	Enhanced sensitivity to ciprofloxacin (double mutant)
	cci-AHL	Enhanced sensitivity to SDS Affected maturation
Candida albicans	Farnesol	Affected biofilm diffusion
Klebsiella pneumoniae	AI-2	Slow development of biofilm
Pseudomonas aeruginosa	las-3 oxo-C12 HSL	Affected biofilm structure e.g. reduced thickness and more sensitive to SDS (las)

	rhl- C4 HSL	Increased sensitivity to hydrogen peroxide and
Serratia liquefaciens	swr- AHL	Affected thickness of biofilm
Serratia marcescens	swr- AHL	Affected biofilm diffusion and re-colonization

1.5 Correlation of QS, Biofilm formation and Antimicrobial resistance (AMR):

1.5.1 Antimicrobial resistance:

Antibiotics have become an integral part of modern healthcare since their introduction into medicine in 1940. However, WHO's 2014 report on global investigation of antibiotic resistance stated that the world is entering into the post-antibiotic era in which infections which were curable with antibiotics for decades are again becoming incurable[23]. Antibiotic resistance is a current challenge to global public health that requires action from every country. The Joint Programming Initiative on Antimicrobial Resistance (JPIAMR) signed by 27 countries is an example of global efforts aimed at reducing the emerging risk of antibiotic resistance and preserving antibiotics for future generations[24]. Antibiotic resistance reduces the efficacy of the treatment and hinders the control of infectious diseases[25]. In 2010, a study done in New Delhi revealed that 24 % of bacteria are resistant to intravenous antibiotics "carbapenems," which act as hospitals' last-resort. 13 % were found to possess the resistant gene, "New Delhi Metallo-beta-lactamase 1," which offers resistance to carbapenems and 14 other antibiotics [25,26]. Advance medical treatments such as organ transplants, joint replacements are hooked on the antibiotics to cure infections occurred during these treatments. Therefore, people undergoing above treatments are at excessive risk, as infections caused by resistant microorganisms do not get cure by traditional antibiotics[27].

1.5.2 Primary reasons of antibiotic resistance:

There are three mechanisms of antibiotic resistance intrinsic, adaptive and acquired[28,29]. Almost all bacteria possess inherent ability to resist some class of antibiotics. This mechanism is called an intrinsic resistance. It is present in the

bacterial genome, and it does not depend on environmental conditions. Conversely, adaptive resistance is developed in response to adverse environmental conditions such as antibiotic selective pressure[29]. This mechanism of resistance is not clear entirely and under ongoing investigation. However, Acquired resistance is a result of the spontaneous chromosomal mutations or gene acquisition through horizontal gene transfer (HGT) via a process of bacterial recombination (transformation, conjugation, and transduction), via mobile genetic elements MGEs (plasmids, integrons, transposons, and genomic islands), or via membrane vesicles and nanotubes[29,30].

1.5.3 Biofilm and antibiotic resistance:

Unicellular bacteria form a unique multicellular structure where they are embedded in the self-secreted extracellular polymeric matrix or proteinaceous material referred to as biofilm[31]. Mechanisms by which biofilms formed by bacteria directly or indirectly leads to antibiotic resistance are given below.

The failure of the antibiotics to enter the biofilm:

The rate of penetration of antibiotics through biofilm depends on the bacterial species in biofilm, biofilm architecture (such as thickness, composition) and also on the antibiotic used[32]. Recently, Tseng et al. (2013) observed that the antibiotics with positive charge- tobramycin did not penetrate the biofilm. Based on this observation they concluded that the antibiotic with positive charge must have interacted with biofilm matrix components having negative charge leading to reduced biofilm penetration[33]. Similarly, Beer et al. (1994) reported that commonly used disinfectant chlorine did not penetrate through mixed biofilm produced by *K. pneumoniae* and *P. aeruginosa*[34]. Suci et al. (1994) performed infrared spectroscopy and showed that ciprofloxacin transport to the colonized surface was lesser than to a sterile surface, suggesting the binding of ciprofloxacin to the biofilm components[34]. Hoyle et al. (1992) used another approach, they developed *P. aeruginosa* biofilms on one side of a dialysis membrane and penetration of piperacillin through the biofilm was measured and results obtained were in accordance with the assumption that biofilm act as barrier to the antibiotic penetration. Thus, variety of methods, such as equilibrium dialysis experiments, diffusion-cell bioassays, sandwich-cup methods, fluorescent tracers or susceptibility testing in media containing slime were used to confirm the effect of penetration failure in drug resistance[32]. However, few experiments also

showed that blocking penetration of the antibiotic is not the only mechanism through which bacteria attains resistance in antibiofilm[32–34].

Slow growth and the stress response:

Bacterial cells under stress condition get embedded within the biofilm and enter a resting state, or dormant state referred as persistence wherein they become resistant to almost all antibiotics without any genetic change[35]. Tuomanen et al. (1986) showed that the rate of bacterial growth and rate of occurrence of antibiotic resistance are inversely proportional[34]. The reason is, most of the antibiotics target basic metabolic pathway of bacteria, e.g. β-lactams target cell wall synthesis in actively dividing bacteria, quinolones introduce nicks during replication therefore at slow growth rate antibiotic efficacy get reduced[28]. Later, research done by Brown et al. (1988) and Wentland et al. (1996) revealed that bacterial growth is reduced within the biofilm[34]. Their result clearly implies that slow bacterial growth within the biofilm leads to antibiotic resistance, and same has been repeatedly proved by many researchers and has been mentioned in many recent relevant reviews[28,35–38].

Heterogeneity and proximity

Many times bacterial biofilms are of poly-microbial nature. Therefore, every cell within biofilm has a slightly different location, gradients of nutrients and signaling factors and therefore different growth rate. This tremendous heterogeneity leads to the inefficacy of antibiotics[34].In addition to this, polymicrobial nature and proximity also enhances the chances of acquired antibiotic resistance through horizontal gene transfer (section 1.5.2).

Some examples of representative bacteria and their tolerance to the antibiotic in the biofilm are mentioned in table taken from reference[39]. Tolerance factor (*TF*) mentioned in table indicates that biofilm killing is 'TF' times slower than in the planktonic condition and it is calculated by formula,

$TF = (LR_P{}^{*} t_B{}^{*} C_B / LR_B{}^{*} t_P{}^{*} C_P)$

Where, C_P and C_B = planktonic and biofilm dose concentration, respectively,

t_P and t_B = planktonic and biofilm dose duration, respectively,

LR_P and LR_B = the measured log reduction in planktonic and biofilm populations, respectively.

Table 1.2: Some examples of representative bacteria and their tolerance to the antibiotics

.

Organisms	**Agent**	***TF***
Propionibacterium acnes	Rifampin	4
	Daptomycin	16
	Vancomycin	16
	Penicillin G	2
Corynebacterium urealyticum	Ciprofloxacin	2048
	Moxifloxacin	512
	Vancomycin	512
Pseudomonas aeruginosa	Gentamicin	4
	Tobramycin	4
	Ciprofloxacin	8
	Ofloxacin	4
P. aeruginosa	Tobramycin	4.4
	Ciprofloxacin	3.5
K. pneumoniae	Ciprofloxacin	90
	Ampicillin	14
Staphylococcus epidermidis	Ciprofloxacin	14
	Rifampin	7
P. aeruginosa	Tobramycin	265
	Ciprofloxacin	104
P. aeruginosa	Tobramycin	208
S. aureus	Nisin	5.3
	Vancomycin	55
S. epidermidis	Levofloxacin	12
	Vancomycin	157
Porphyromonas gingivalis	Amoxicillin	3.3
	Doxycycline	21
	Metronidazole	4.2
Staphylococcus lugdunensis	Cefazolin	256

	Rifampin	4
	Daptomycin	64
	Moxifloxacin	4
	Naficillin	16
Candida albicans	Amphotericin B	3.4
C. albicans	Fluconazole	4.4

As quorum sensing mediate biofilm formation phenotype, inhibiting quorum sensing in pathogenic organisms could be a novel and a preventive approach to decrease the global burden of antibiotic resistance and thus severe pathogenesis[40,41].

1.6 Can bacteria become resistant to QSIs like conventional antibiotics?

Conventional antibiotics' activity is either bactericidal or bacteriostatic. They target fundamental processes like replication, DNA repair, bacterial cell wall synthesis or synthesis of the protein. Hence, put selective pressure on bacteria and spreads resistance very rapidly and to such extent that they eventually start showing resistance to nearly all antibiotics under any environmental conditions[40,42,43] However, many reports have shown that unlike antibiotics QSIs exclusively target quorum sensing in bacteria without affecting their cellular processes required for survival. Therefore, bacteria are less likely to develop resistance to QSIs[40,42,43].

Gerdt et al. (2014) thoroughly evaluated barriers that may prevent the resistance to QSIs. He used QS mutants of *P. aeruginosa*. Their competition study confirmed that the development of resistance to QSIs will not be possible because of two significant reasons. First, though QSI resistant bacteria inhabiting in the group of QSI susceptible bacteria will synthesize signal molecules, the concentration of the signals will not be sufficient to trigger quorum sensing. Therefore will not have a fitness advantage over QSI sensitive bacteria and has no chance for QSI resistance. Second, suppose few QSI resistant bacteria will breach the first barrier and initiate expression of QS controlled genes then such resistant bacteria are vulnerable to cheating by other QSI sensitive bacteria. The cheaters will take benefit from the common goods (such as proteases, siderophores), but they will not respond by producing the group beneficial goods[44].

Thus, QS inhibition strategy has significant potential as resistance-robust therapeutics.

1.7 Quorum quenching mechanisms:

The different quorum sensing disruption mechanisms are discovered so far Briefly, (1) Inhibition of signal binding to receptor, (2) Blocking of signals, (3) Inhibition of signal synthesis, (4) Suppression of LuxI –family synthase and LuxR-family receptor synthesis and their activities and (5) Degradation of signals[7,45,46] (Figure1.7).

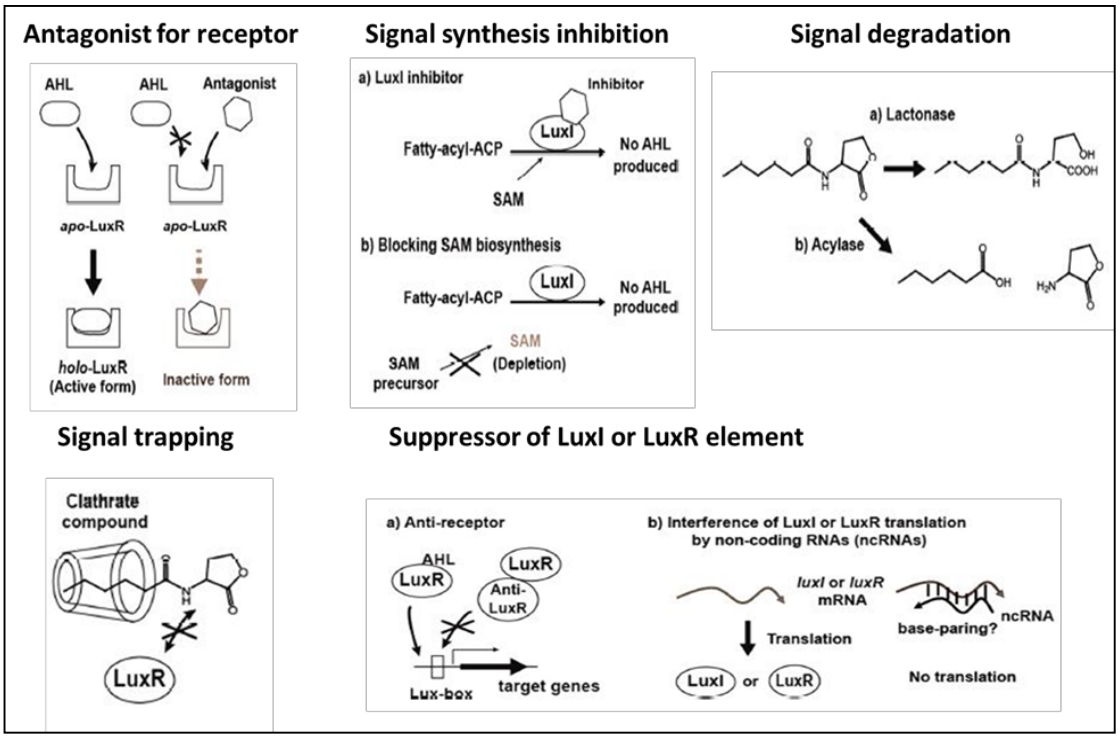


Figure 1.7: Proposed strategies for AHL quorum sensing inhibition. Figure adapted from reference[45]

1.8 Applications of QSI:

1.8.1 Aquaculture:

Area of aquaculture has increasingly developed recently being an essential source of food. However, it is always escorted with frequent occurrence and re-occurrence of several infectious diseases caused by opportunistic pathogens e.g. *Vibro sp*., *Pseudomonas sp*., and *Aeromonas sp*. These disease outbreaks are routinely treated with antibiotics, and have caused development of multiple drug-resistant

bacteria. As pathogenesis of these bacteria is mainly triggered by quorum sensing mechanism, use of QSIs has great prospective for sustainable aquaculture with the reduction in antibiotic usage in this field[47,48]. Some representative QSI molecules which have been proved to inhibit pathogenesis in aquaculture are mentioned in Table 1.3

Table 1.3: Representative QSI molecules and their activity against aquaculture pathogen

QSI source /molecule	**Host**	**Pathogen**	**Ref.**
Brominated furanone	Rainbow trout — Oncorhynchus mykiss	*V. anguillarum*	[49]
	Artemia	*V. para-haemolyticus*, *V. harveyi BB120, V. campbellii* and	[50]
	Rainbow trout — *Oncorhynchus mykiss*	*V. harveyi*	[51]
	Prawns	*V. harveyi*	[52]
Shewanella sp	Ayu fish	*V. anguillarum*	[53]
Phenerylamide metabolites from Marine *Halobacillus salinus* strain	-	*V. harveyi*	[54]
AHL-degrading bacteria, *Bacillus* spp	Turbot- *Scophthalmus maxima*	-	[55]
N-(heptylsulphanylacetyl)-L-homoserine lactone (HepS-AHL)	-	*P. aeruginosa*	[56]
N-tetradecanoyl-L-homoserine lactone effectively	Burbot larvae	*A. hydrophila* and *A. salmonicida*	[57]

3-(methoxyphenylpropi-onamido) ribofuranosyl derivative	*Artemia* sp.	*V. harveyi*, *V. anguillarum*, *V. vulnificus*	[58,59]
4-methoxycarbonyl-phenylboronic acid (MCPBA, an AI-2 homologue) and Pyrogallol	-	*V. vulnificus*, *V. anguillarum*	
Cinnamaldehyde	*Artemia* sp.	*V. harveyi*, *V. anguillarum* and *V. vulnificus*	[60]
	Burbot larvae	*A. hydrophila* and *A. salmonicida*	[61]
Thiophenone compound TF310, (Z)-4-((5-(bromomethylene)- 2-oxo-2,5-dihydrothiophen-3-yl) methoxy)-4-ox-obutanoic acid	*Artemia* sp. larvae	*V. harveyi*	[57,62]
Chlamydomonas reinhardtii and the micro-algae *Chlorella saccharophila*	Burbot larvae	*A. salmonicida*, *A. hydrophila*	[57,61]
Curcumin	-	*V. parahaemolyticu*, *V. vulnificus* and *V. harveyi*	[57]

1.8.2 Wastewater treatment:

Nowadays, biofouling of membrane filters of membrane bioreactors (MBRs) is a significant challenge in front of wastewater treatment technology. Biofilm formation cause transmembrane pressure development and disturbs the permeability of the membrane. This causes higher treatment cost. Use of QSIs in membrane bioreactor has great potential to reduce biofouling in MBRs[47,63,64]. Some recent examples of QSIs used in MBRs are mentioned in Table 1.4

Table 1.4: QSIs in a membrane bioreactor

QSI	Activity	Ref.
Porcine kidney acylase I enzyme	Inhibition of biofouling in MBR	47
Immobilized porcine kidney acylase I enzyme	Increased anti-biofouling efficiency of MBR	47
Quorum quenching (QQ) cylinders (moving QQ cylinders)	Increased anti-biofouling property	65
Polymer beads-entrapping *Candida albican*s (a farnesol producing fungus)	Regulation of biofouling and specific aeration energy savings	66
QQ bacteria activated with Gamma caprolactone	Lower AHL in the surrounding, higher QQ activity, regulate the secretion of extracellular polymeric substances (EPS), reduced biofouling in an MBR	67
Acinetobacter sp. DKY-1	Reduced membrane biofouling.	68
Lactobacillus sp. SBR04MA	Reduced biofouling, enhanced MBRs' performance	69
QQ sheets	Inhibition of biofouling in pilot-scale MBR	70

1.8.3 Biotransformation:

In this process, there is a need of dispersal of biofilm and recycling of the platform for next batch of biotransformation. Therefore, like membrane bioreactors (as mentioned in section 1.8.2) QSIs (biological or synthetic compounds, quorum quenching (QQ) bacteria-as dispersal cell) can be used[47].

1.8.4 Plant cultivation:

Ralstonia, *Pseudomonas*, *Erwinia*, *Xanthomonas*, and some *Streptomyces spp*., are the few major genera of pathogenic organism causing severe plant diseases such as spots, bacterial blight, pustules on leaves and fruits, rot, or mosaic patterns[71]. As per some recent studies, quorum sensing inhibitors can be used to prevent plants from various diseases. It has been found that *Microbacterium testaceum* an endophytic bacterium present on the surface of potato leaf possess an AHL degrading property with which it protects the plant from soft rot disease of *Pectobacterium carotovorum*[72]. Engineering the plant or plant associated microbe producing QQ enzymes could help in crop protection. Based on this assumption, genetically modified plants produced by integrating lactonase into tobacco plants, potato, cauliflower, Chinese cabbage, and synthesis of Aiia enzyme (which inactivates the AHL signal in *Erwinia carotovora*) resulted in increased resistance to plant diseases[73,74]. Similarly, the expression of AHL degrading enzyme encoded by aiiA in the insecticide *Bacillus thuringiensis* resulted in the strain inhibiting QS mediated pathogenesis in AHL-dependent pathogen *E. carotovora* [75]. The same aiiA gene was also introduced in the QS pathogen *Burkholderia glumae*, causing rice grain rot and it was observed that AHL concentration in the transformants was reduced significantly and blocked *B. glumae* pathogenesis. Similar results were also observed in the strain *P. aeruginosa* expressing QQ enzyme[71]. Teplitski et al. (2000) observed that some plants like *Medicago truncatula* and *Pisum sativum* can produce QS mimics upon bacterial infections and these AHL analogues help to defend plants from pathogenic bacteria by attenuating virulence[76].

1.8.5 Human health:

QSI potential of garlic has been demonstrated through five methods, first *lasB-gfp* (ASV) QS monitor assay where 2 % concentration of garlic significantly reduced

synthesis of GFP and decreased induction by two-fold, second through DNA microarray analysis, third by observing growth of biofilm produced by *P. aeruginosa*, fourth by studying tolerance of biofilm to tobramycin in the presence of garlic extract and fifth by using *Caenorhabditis elegans* nematode model[77]. Further, it was also found that the prior treatment with garlic enhances susceptibility of biofilms to tobramycin and phagocytosis by PMN. In addition to this, garlic extract also showed improvement in clearance of pulmonary infection[78]. So based on this preliminary work on QSI activity of garlic extract, the first clinical trial for treating cystic fibrosis patients has been carried out by Prof. Givskov and his team[79]. In this trial, 656 mg/day of garlic oil was given to 26 patients for 8 weeks. Results observed were very encouraging in terms of functioning of lung but as results were not significant therefore Prof. Givskov and his team suggested that such research should be done on higher scale[79].

In addition to this, QSIs can be used in various other areas, e.g. surface coatings of ship hulls, water pipes, tanks, reactors, food packaging, dental implants, surgical implants, and catheters to reduce biofouling and for improving the efficiency of QSI, their nano-formulations can be made[80]. Large scale production of QQ enzymes can be achieved through incorporating such gene complex in non-pathogenic quorum sensing bacteria[47].

1.9 Current scenario of industrial production and application of QSIs

Need of QSIs can be understood from an escalating number of patents and research papers in this field. Patent information collected from freepatentsonline.com showed that 1524 patents based on quorum sensing inhibitors have been filed[7]. However, none of the quorum sensing inhibitors identified so far have reached the market yet. This indicates the requirement of further basic research and initiative from pharmaceutical companies for developing QSI as a drug molecule. As mentioned earlier (in section 1.8.5) clinical trial of garlic oil by Prof. Givsko was the first step towards commercialization of QSI molecule[79].

Few pharmaceutical companies like GlaxoSmithKline (GSK) are on their toe for developing innovative ways to solve the problem of antimicrobial resistance. Some start-ups are also operating in the arena of QSI or using QS as a key thing for any application e.g. Quorex Pharmaceuticals Inc. Carlsbad, California, the USA is a

company which carries out production of quorum sensing inhibitor. It was founded in 1999 by Bonnie Bassler, Jeffrey Stein, Robert Robb, and Michael Surette. Quorex targets the conserved AI-2 quorum sensing system present in Gram positive as well as Gram negative bacteria[81,82]; Aurora Biosciences, Iowa City, IA. company was founded in 1995 and acquired quorum science in 2000[81]; QSI Pharma A/S, Lyngby, Denmark was founded in 2002 by the Technical University of Denmark, and the biotech incubator of Leo Pharma AS. QSI pharma aims to develop molecules interfering with bacterial quorum sensing. More emphasis is given on *Pseudomonas aeruginosa* QS[83]; Microbia, Cambridge, Massachusetts, USA[84]; QuoNova ,US. was developed in 2006 by the drug innovation and improvement company 4SC AG (Frankfurt), located in Martinsried, Germany and XL TechGroup, Inc. (AIM: XLT) located in Florida US. This company works on synthesis of QSB (quorum sensing blockers) and substances which influence bacterial biofilm formation[85]; Selenium, Ltd., was established in 2004 through joint innovation by Dr. Reid T. and Dr. Spallholz J. from Texas Tech University, USA and funded by venture-capital firm Emergent Technologies. It is mainly involved in the production of selenium-based surface coatings like Seldox products are used to provide anti-adhesive surface coatings for medical devices such as contact lenses, catheters, voice prostheses; and Curza a small molecule therapeutic company started in 2016 in Salt Lake City, USA is engaged in development of novel coatings to avoid biofilms on knee as well as hip implants[86].

Though many companies have started as mentioned above, big pharmaceutical companies are yet to draw positive attention to QSIs. QSIs have showed their effectiveness in lab as well as in many animal models but still more experiments are needed for successful clinical trials and for the genuine marketing so as to benefit common man[7]. Considering the enormous applications of QSIs, it is essential to understand the structure and the mechanisms of action of QSIs. This could help in the designing of superior QSIs for various applications. Even if large numbers of QSI patents are available, they may have some constraints during *in vivo* conditions. QSIs which are AHL analogues may undergo hydrolysis of lactone ring and protein based QSIs may encounter stability problems. For example, though many AHL analogues have been discovered as QSIs, it may not withstand lactonases produced in the human epithelia. Therefore, extreme care is required in the development of AHL analogue

based QSI having lactone core. QSI drugs which will withstand lactonase can be designed to evade this problem[7]. At present, the high specificity of QSI drugs limits their application, especially against microbial communities as these drugs may not act on closely related species. The consequence is that narrow spectrum compounds may not come to market because of their low efficacy.

Along with broad specificity QSI compounds' stability within the biofilm matrix is also important[87]. For the compound to be QSI molecule it is important that it specifically target pathogen without killing them and it does not affect the host adversely. Unfortunately, for some of the reported compounds, this aspect has been ignored but evaluation of their cytotoxicity is mandatory for successful commercialization. Biocompatibility is an essential aspect for QSI compounds. Drug release at specific location could be an option particularly for biofilm-inhibting compounds on implants[87]. The lack of proper delivery systems is one challenge in using QSI compounds in a medical formulation, so nano-formulations of QSI can be prepared[80]. Thus, it is more important to work on deciphering basic mechanisms of QSI to find its effect on the host organism and *in vivo* stability of QSI compounds than the discovery of novel compounds with similar activity[88].

1.10 Prerequisites of ideal quorum sensing inhibitor

Considering the current requirement of QSI field, potent QSI should be screened based on the below-mentioned criteria.

1. It should be a low molecular weight compound.
2. It should be highly specific towards its target, e.g. QS regulators (LuxI/R type proteins)
3. It should be biocompatible and should not be toxic to either eukaryotic host or bacteria i.e. should not impose selective pressure by affecting basic cell processes of a bacteria-like synthesis of the cell wall, DNA and protein.
4. QSI should be stable and resilient to metabolism and disposal by the eukaryotic host.
5. It should be either readily available or should have an easy method of synthesis[7,89].

1.11 Essential oils as quorum sensing inhibitors:

Exploring medicinal uses of natural products is a flourishing trend nowadays due to fewer or no side-effects than allopathic medicine, better patient tolerance, and relative inexpensiveness[90]. Among all the natural phytoproducts, essential oils have been widely used in the treatment of various diseases. Essential oils also known as volatile oils are natural, complex, multi-component, aromatic liquids extracted from plant material. Essential oils are widely used due to their bactericidal, virucidal, fungicidal, antiparasitical, insecticidal properties. Essential oils are used in pharmaceutical industry for medicinal preparations like ointments, toothpastes, tooth powders, and mouthwashes; in paint industry; in food and beverage as preservatives; in adhesives such as glue and cements; in textile industry for finishing deodorant; in leather tanning industry as preservatives; in petroleum industry for oil waxes solvent, lubricating creams[91–94]. Interestingly, some recent *in-vitro* studies have also found that essential oils can reverse antibiotic resistance in bacteria, but still some basic studies are required to avoid the probability of bacterial resistance to essential oils in the future[90]

Apart from all above-mentioned properties of essential oils, they are also reported as quorum sensing inhibitors. Some representative examples are mentioned below Table 1.5.

Table 1.5: Essential oils as quorum sensing inhibitors

Essential oil	**Activity**	**Ref.**
Coffee husk oil from *Coffee arabica L.*	Inhibited QS controlled phenotypes such as violacein pigment production (in *C. violaceum* CV026) and elastase, pyocyanin, EPS and biofilm (*in P. aeruginosa*)	[95]
Essential oils from *Piper bredemeyeri*, *Piper brachypodom* and *Piper bogotence*	All inhibited violacein in *C. violaceum* CV026	[96]
Essential oils from *Cuminium*	Inhibited QS controlled	[97]

cymium, *Murraya koenigii*, *Curcuma longa*, *Zingiber officinale*, *Myristica fragrans*, *Trigonella foenum-graecum* and *Elettaria cardamomum*.	phenotypes such as violacein (in *C. violaceum* CV026), *C. violaceum12472*, Pyocyanin, pyoverdine, swimming swarming and twitching motility and biofilm (*in P. aeruginosa*)	
Clove oil	Significant reduction of LasB, total protease, chitinase and pyocyanin production, swimming motility, exopolysaccharide and biofilm production in *P. aeruginosa* and *Aeromonas hydrophila*	[98]
Cinnamon oil	Inhibited violacein (in *C. violaceum* CV026); and 3-oxoC12 production, pyocyanin, swarming, alginate, protease and biofilm (in *P. aeruginosa)*	[99]
EO components - carvone, hexanal, carvacrol, citral, geraniol, salicylic acid, cinnamic acid, thymol, eugenol and cinnamaldehyde	Violacein inhibition in *C. violaceum* CV026; inhibition of biofilm, motilities and EPS production in *E. carotovora* and *P. fluorescens*	[100]
Clove, bay, pimento berry oils and major component eugenol	Inhibited *Escherichia coli* O157:H7 EHEC biofilm formation, down-regulated genes required for biofilm attachment and maturation curli genes (*csgABDFG*), type I fimbriae genes (*fimCDH*) and *ler*-controlled toxin genes (*espD*, *escJ*, *escR*, and *tir*)	[101]

Essential oils of *Syzygium aromaticum* and *Cinnamomum zeylanicum* (eugenol and cinnamaldehyde)	Reduced biofilm formation on polystyrene and on stainless steel surfaces	[102]
Essential oils from *Lippia alba*	Violacein pigment inhibition in *C. violaceum* CV026	[103]
Green cardamom essential oil	Violacein pigment inhibition in *C. violaceum* CV026	[104]
Ferula asafoetida essential oil	Violacein inhibition in *C. violaceum* CV026 and pyocyanin inhibition in *P. aeruginosa*	[105]
Essential oils of *F. macedonica* and *E. sibthorpiana*	Inhibited biofilm formation, pyocyanin production, twitching and flagella motility	[106]
Murraya koenigii essential oil	Inhibited pyocyanin ,proteases production ,and increased survival of *C. elegans*	[107]
Syzygium aromaticum (Clove) oil	Inhibition of violacein production and reduction in swarming motility in *P. aeruginosa* due to a-caryophyllene and b-caryophyllene components	[108]
Peppermint essential oil	Inhibited luminescence produced by *E. coli* pSB1075	[109]
Peppermint essential oil	Inhibited quorum sensing mediated through AHL and inhibited biofilm formation by *P. aeruginosa* and *A. hydrophila*	[110]
Oregano essential oil and pectin-Oregano films	Inhibited QS in food associated microorgansism	[111]
Essential oils containing alpha pinene, beta pinene, cineol, alpha zingiberene and pulegone	Inhibited short chain AHL quorum sensing (QS) system	[112]

Citrus (Pompia and grapefruit) EOs	Inhibited quorum sensing in *P. aeruginosa* and inhibited biofilm formation by both *P. aeruginosa*, *Candida albicans*.	[113]
Rose, Clove, Chamomile essential oils	Inhibition of violacein in *C. violaceum* CV026 and pyocyanin inhibition in *P. aeruginosa*	[114]
Rose, geranium, lavender and rosemary oils, eucalyptus and citrus oils	Inhibition of violacein in *C. violaceum* CV026	[115]
a-Terpineol , cis-3-nonen-1-ol	>90 % violacein inhibition	[116]
Glycolipids of essential oils (Lemongrass oil, Peppermint oil, Cinnamon oil, Rosemary oil, Basil oil, Bergamot oil, Eucalyptus oil, Orange oil, Citronella oil, Tea tree oil, Ylang ylang oil, Frankincense oil),	Inhibition of violacein (in *C. violaceum* CV026) and biofilm inhibition (by *P. aeruginosa*)	[117]
Eugenol, cinnamaldehyde and vanillin, *Ferula asafetida* (ferula) oil and *Dorema aucheri* (dorema) oil	Inhibit quorum sensing	[41]
Thymus vulgare (Lamiaceae) and *Centella asiatica* (Apiaceae	Inhibit biofilm formation by *Pseudomonas sp*. and pigment production in *Chromobacterium violaceum* ATCC12472	[118]

1.12 Limitations of essential oils:

Though many essential oils are reported as QSIs none of them have reached till clinical trial. This could be attributed to hydrophobicity and complex nature of essential oils. Since essential oils are highly hydrophobic medicinal formulation containing only essential oil may lead to sensitization, irritation, skin burns, mucosal damage[119]. Recent studies have suggested that along with novelty, the solubility of drugs is equally important to achieve therapeutic efficacy and to capture market

economies. Therefore, drug solubility enhancement has become a critical challenge in front of pharmaceutical companies[120]. As per classification of Bio-pharmaceutics Classification System (BCS), 4 classes of drugs are present out of which Class II and IV categories (high/low permeability and low solubility) need modifications to achieve therapeutic excellence[121]. Solubility enhancement of essential oils is required to overcome mucosal barrier, to attain appropriate concentration of drug in systemic circulation for getting desired response, to reduce dose of the drug, to avoid non uniform distribution and to overcome sensitization, irritation, skin burns, mucosal damage. (Detailed classification system and importance of solubility of drugs are mentioned in Chapter 2a) [121–125]

1.13 Solubility enhancement techniques:

These techniques are majorly categorized into two, physical modification, chemical modifications and in addition to these two categories some other techniques are also available.

1. Physical modification Techniques: Particle size reduction (micronization and nanosuspension), modification of the crystal habit viz. polymorphs, amorphous form and cocrystallization, drug dispersion in carriers, e.g. eutectic mixtures, solid dispersions, solid solutions, and cryogenic techniques.
2. Chemical modification techniques: Change of pH, use of buffer, derivatization, complexation, and salt formation.
3. Other techniques: Supercritical fluid process, use of adjuvant like surfactant, solubilizers, co-solvency, hydrotrophy, and novel excipients[122].

Nowadays, in the pharmaceutical industry for improving solubility of the drug, physical modification techniques are commonly used. However, methods like, hot melt extrusion includes exposure to shear and high temperature for a relatively longer duration which may affect the stability of the drug[122,126]. Essential oils are commonly used with an emulsifier to increase its solubility. However, choice of appropriate emulsifier, its composition, and high molecular weight are significant issues in the application. Conversely, surfactant's low molecular weight and emulsifying activity contribute to wide-ranging industrial uses[127,128].

1.14 Biosurfactant–sophorolipids as solubility enhancer

Recently, biosurfactant-sophorolipids (SLs) are under investigation for enhancing the solubility of a drug due to their myriad advantages viz. high biodegradability, low toxicity, excellent biocompatibility, easy method of synthesis, more stability and digestibility. SL is an amphiphilic molecule and synthesized by non-pathogenic yeast *Candida bombicola*. SLs have hydrophilic portion consist of glucose disaccharide called as sophorose unit with a unique β-1, 2 bond and hydrophobic part consist of fatty acid tail which is bound to 1' hydroxyl group sophorose unit with either the penultimate or terminal carbon atom of the fatty acid chain. Sophorolipids are synthesized in the form of a mixture of both acidic and lactonic sophorolipid. In case of an acidic form, the carboxylic end of the fatty acid is free, whereas, in lactonic form, it is esterified at the 4″ or in some exceptional cases at the 6′ or 6″ position which adds variations in sophorolipid structure (Figure 1.8) [129].

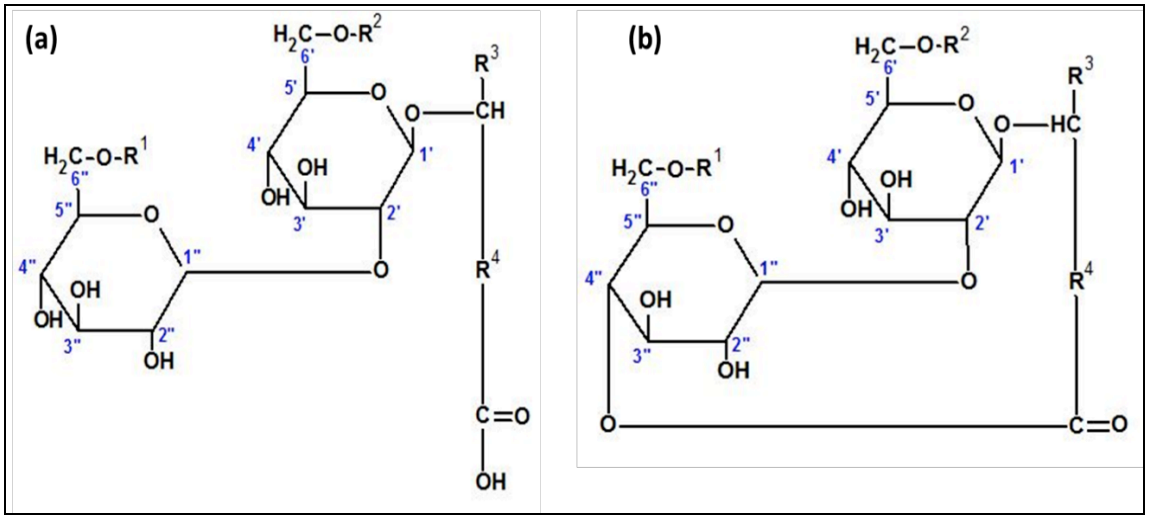


Figure 1.8: Basic sophorolipid structure: (a) Acidic form (b) Lactonic form

Sophorolipids possess one unique property of being tailor-made, i.e. apart from conventional C16-C18 fatty acids other fatty acids can be incorporated to get variant of SL. Thus this property will broaden up the application potential of SL. There are two reported strategies to avoid specificity of C16-C18 fatty acids. The first one escape action of cytochrome P450 mono-oxygenase enzyme by supplementing already hydroxylated lipophilic substrate, however other method is to use substrate structurally similar to stearic acid. In the present work, we are using later strategy.

In our study for the first time, *C. bombicola* was supplemented with non-conventional lipophilic substrate monoterpene aldehydes citral and citronellal- two major constituents of lemongrass oils. The rationale behind choosing lemongrass oil is its quorum sensing inhibitory activity[117] However, lemongrass oil is a complex mixture of volatile compounds and each compound of essential oil may have various targets. Therefore, major components citral and citronellal were used instead of whole essential oil to avoid the complexity of converted compound[130].

1.15 Citral and citronellal -major components of essential oil:

Citral and citronellal are hydrophobic monoterpene aldehyde usually isolated from plants of Poaceae (grass) family *Cymbopogon citratus* and *Cymbopogon nardus* respectively[131,132]. Espina et al. (2015) have shown that citral can inhibit biofilm produced by *Staphylococcus aureus*[133], Shi et al. (2017) have reported that citral can inhibit expression of virulence factors in *Cronobacter sakazakii*[134]. However, Colorado et al. (2012) have found that citral can inhibit quorum sensing mediated through a short chain and long chain AHL molecule[112]. Still, real potential of citral is not yet explored due to hydrophobic nature. On the other hand, antiquorum sensing activity of citronellal has not reported so far**.**

1.16 The scope of the work

Although many reports on QS inhibitors have been published along with around 1000 patents, yet none of them has been commercialized as QS inhibitor [135,136]. Many essential oils which are reported as QSIs come in the above-mentioned category (Table 1.5). Although essential oil possesses quorum sensing inhibitory activity, their hydrophobicity and multiple components limits their applications. Therefore, methods which will modify essential oil itself to biocompatible form without affecting their biological activity are highly desirable for their use in medical preparations.

Primary focus of my research was to develop highly efficient, stable as well as biocompatible quorum sensing inhibitory molecule which will have immediate applications in the future. The main aim of my research was to convert a major component of essential oil to hydrophilic form with increased bioavailability and to check quorum sensing inhibitory activity of the converted compound. Another

objective was to find its mode of action at the molecular level to use it in the areas where biofilm-associated problem is a major challenge.

1.16.1 Synthesis, characterization and QSI activity of G-citral and G-citron

Conversion of monoterpene aldehydes to glycomonoterpenes was confirmed by TLC, HPLC, FTIR, MALDI, and NMR. As there were reports on QSI activity of lemongrass oil, we explored QSI activity of G-citral and G-citron synthesized from major components of lemongrass oil citral and citronellal respectively by using two quorum sensing reporter strains *Chromobacter violaceium* - CV026 and *Agrobacterium tumefaciens* -Ag(pZLR4). Quorum sensing mediates various phenotypes as mentioned in Figure 1.5. Therefore, the effect of G-citral and G-citron on biofilm formation and virulence factor production was also studied. After confirming QSI activity of glycomonoterpenes, we have found out mode of action of G-citron in quorum sensing pathway of *P. aeruginosa.* Further two applications of G-citron (QSI) in implant coating and contact lens cleaning solution was studied.

1.16.1 G-citron (QSI) in implant coating:

Many groups of researchers from worldwide are focused at finding most appropriate quorum sensing inhibitors (QSI) that could be used for implant coating. Kratochvil et al. (2015) have recently prepared, nanoporous superhydrophobic coatings that promote the extended release of water-labile peptidic quorum sensing inhibitors and attenuated quorum sensing in *S. aureus*[137]. This opened new facet for applicability of QSIs in coatings of implants or other surfaces to attenuate bacterial virulence and fouling. However, many challenges remain with respect to flaking, delamination, cracking and crazing of coatings. The doctoral work reported here takes a step towards addressing these challenges through the use of silk fibroin and novel process of PDMS surface coating to promote the long-term release of G-citron (QSI) that modulate quorum sensing.

1.16.2 G-citron (QSI) in contact lens cleaning solution:

More than 50 % of contact lens-associated corneal infections are caused due to bacterial colonization and deposition of bacterial toxins and byproducts on contact lenses. Many research groups are working on reducing biofilm formation on the contact lens. However, none of them have tried the approach of quorum sensing

inhibitory molecule to reduce biofilm inhibition. Therefore, in the work reported here we have demonstrated the use of G-citron (QSI) to increase *P. aeruginosa* biofilm inhibition efficiency of contact lens cleaning solution for a longer duration.

Thesis outline

The thesis is broadly divided into four parts and which is further divided into six individual chapters, as follows:

Introduction

Chapter 1: Strategies for impeding quorum sensing in bacteria: Basic and applied aspects**.**

Synthesis and characterization of QSI

Chapter 2a: Biosynthesis of novel glycomonoterpenes using monoterpenes of essential oils and their characterization

Chapter 2b: Exploring biological activity of glycomonoterpenes (G-citral and G-citron) as quorum sensing inhibitory molecules.

Mode of action of QSI

Chapter 3: Deciphering mode of action of G-citron by studying its effect on quorum sensing related gene expression in *P. aeruginosa*.

Applications of QSI

Chapter 4a: Development of novel G-citron based surface coating for biomedical implants

Chapter 4b: Synergistic effect of G-citron and lens cleaning solution to increase the antibiofilm effect

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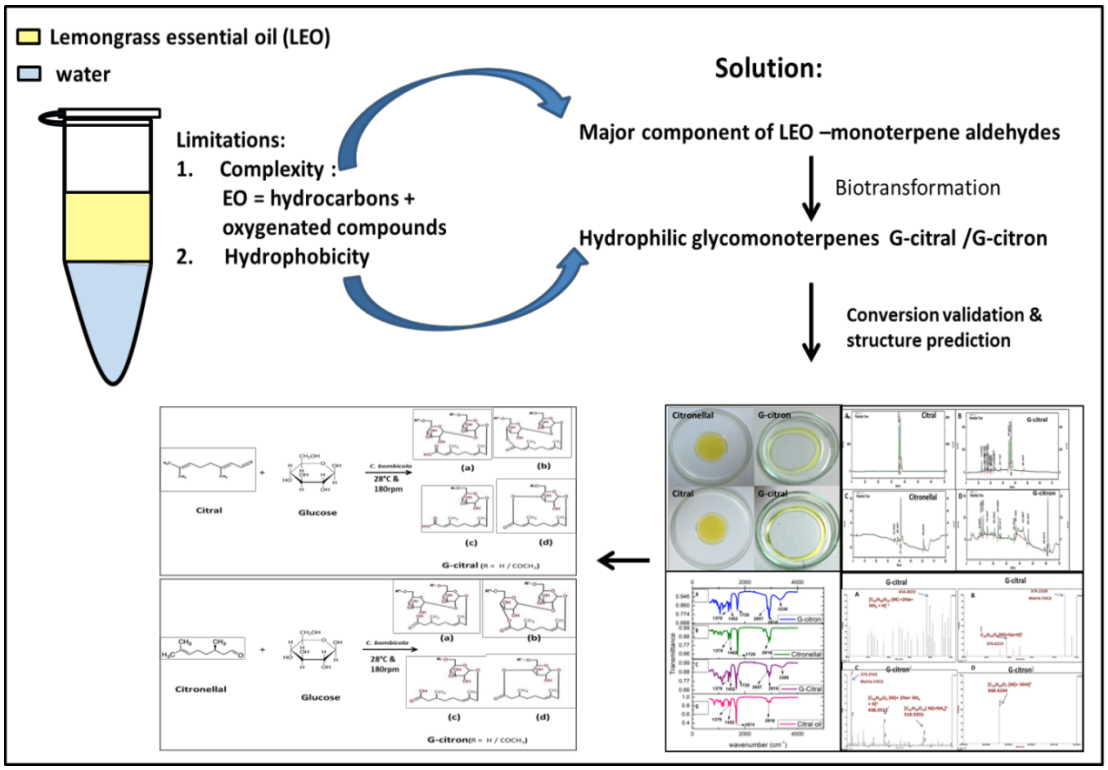
Chapter 2a.

Biosynthesis of novel glycomonoterpenes using monoterpenes of essential oils and their characterization



Recently, plant-derived essential oils have been reported as quorum sensing inhibitors, but biological activities of these essential oils become limited due to their hydrophobic nature. Citral and citronellal are major components of various easily available essential oils such as lemongrass and citronellal, respectively. There are two key problems in using citral and citronellal as quorum sensing inhibitors. First, these monoterpenes are highly hydrophobic, and second, they possess antibacterial activity thus cannot be used as quorum sensing inhibitors as they impose selective pressure on bacteria. Therefore, in the present work to increase bioavailability and to achieve QSI activity, these monoterpenes were converted into glycomonoterpenes. Conversion of citral and citronellal to their respective glycol derivative was confirmed by doing an oil displacement activity, TLC, HPLC, FTIR, NMR analysis of parent and converted compounds, respectively. These interesting findings regarding the conversion of

hydrophobic essential oil components to hydrophilic components add a new facet to the known range of medical applications of essential oil.

2a.1 Introduction

The alarming rate at which antibiotic resistance is spreading has placed whole mankind at the brink of the 'Post antibiotic era'[1]. Antibiotics which were effective earlier are no longer in effect. Though scientists are continuously working to invent new antibiotic; resistant variants are emerging very rapidly[2]. As mentioned in the earlier chapter, chief reasons behind antibiotic resistance in bacteria are biofilm produced by quorum sensing mechanism and selective pressure imposed by antibiotics[3].

Quorum sensing (QS) phenomenon helps bacteria to communicate with surrounding bacterial population through production, release, and perception of the quorum sensing signal molecule called as autoinducer[4]. Quorum sensing inhibitor (QSI) does not impose selective pressure on the target organism as well as will inhibit biofilm formation. Thus, targeting QS is a promising strategy against antibiotic resistance[4,5]. Although the enormous numbers of QS inhibitors have been reported along with around 1000 patents in the last 15 years, very few QSI molecules reached human clinical trials. Therefore, research focus should be on the development of highly efficient, stable and biocompatible molecules which will also retain their efficacy *in-vivo* rather than reporting new quorum quenching molecules[6].

Nowadays, around 88 % of the world's population relies on traditional medicine for their primary healthcare [7]. Among all the traditional medicine, essential oils have been widely used in the treatment of various diseases. Along with medical applications EOs are also used in various industries as shown in Figure 2a.1. Essential oils are natural, complex, multi-component, aromatic liquids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots). They are extracted by using water or steam distillation, solvent extraction, expression under pressure, supercritical fluid and subcritical water extractions. It is called an essential oil because they contain the essence of plant fragrance[7,8].

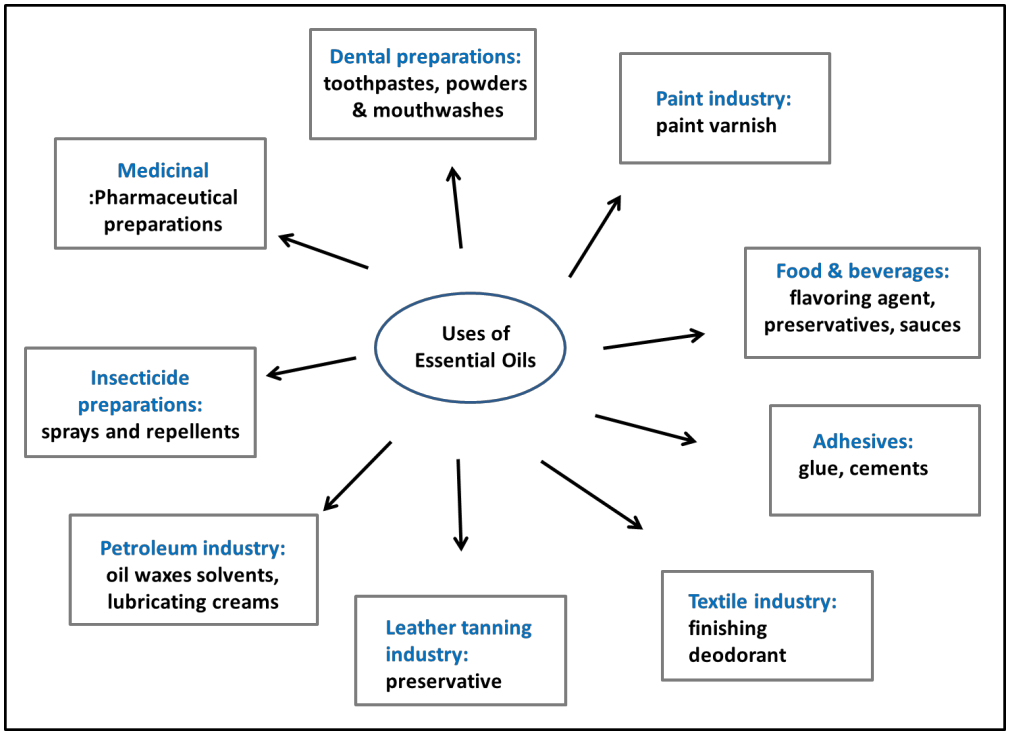


Figure 2a.1: Uses of essential oils in various industries[9–12].

Apart from all above-mentioned properties of essential oils (Figure 2a.1), they are also reported as quorum sensing inhibitors (Table 1.5). However, they are not commercialized as quorum sensing inhibitor due to their hydrophobic nature and complexity.

Essential oils and complex nature:

Essential oils are a complex mixture of volatile compounds. These are comprised of hydrocarbons (monoterpenes, diterpenes, sesquiterpenes) and oxygenated compounds (Esters, aldehydes, ketones, alcohols, phenols, and oxides). Though essential oils are used in various medical applications, being a mixture of many components tracking interactions of each component becomes difficult. So using single component is preferred for therapeutic use to avoid complications of side-effects. In essential oils monoterpenes are combined with Aldehyde (citral, citronellal), Alcohol (geraniol, linalool, menthol, borneol) Ketone (menthone, carvone, thujone), Ester (bornyl acetate, linalyl acetate), Ether (1,8-cineol) or Phenol (thymol, carvacol)[13]. Therefore, in our study citral and citronellal from monoterpene aldehydes category and which are major constituents of lemongrass oils usually extracted from *Cymbopogon citratus* and *Cymbopogon nidus* respectively were chosen to increase solubility (Figure 2a.2). The rationale behind choosing lemongrass

oil is its quorum sensing inhibitory activity[14]. Citral and Citronellal both are hydrophobic monoterpene aldehyde usually isolated from plants of Poaceae (grass) family *Cymbopogon citratus* and *Cymbopogon nardus* respectively[15,16].

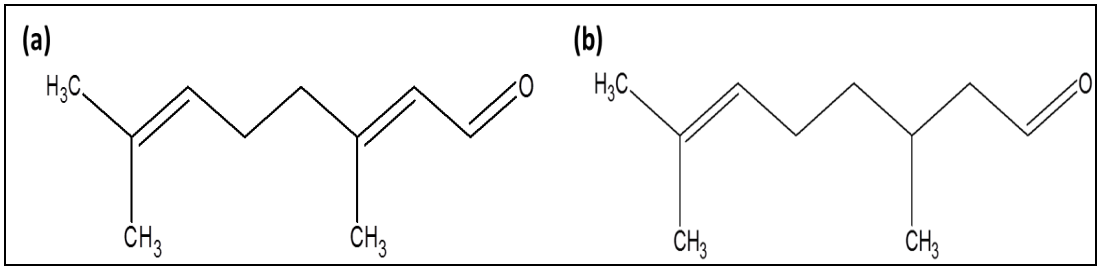


Figure 2a.2: Structure of (a) citral and (b) citronellal

Essential oils and hydrophobic nature:

Recent reports, have suggested that the development of novel drugs alone are not enough to achieve therapeutic efficacy and to capture market economies[17]. Majority of the failures in new drug discovery is due to the poor water solubility of the drug. Therefore, drug solubility enhancement has become a critical challenge in front of pharmaceutical companies. The Bio-pharmaceutics Classification System (BCS) have classified drugs as follows based on solubility and permeability of the drug[17,18]

- Class I: High permeability and solubility
- Class II: High permeability and low solubility
- Class III: Low permeability and high solubility
- Class IV: Low permeability and low solubility [18].

So drugs from Class II and IV categories need modifications to achieve therapeutic excellence

Importance of solubility enhancement [18,19]:

1. To overcome the mucosal barrier: Mucosa is extensively distributed among the human tracts, including the oral cavity, nasal cavity, airways, gastrointestinal (GI) tract, genital tract, and eyes. There are numerous advantages of mucosal delivery of the drug such as perfect for mild administration, reduce treatment time, avoid hepatic first-pass metabolism, increases bioavailability and therapeutic effects. However, for penetration through this mucosal layer drug should be hydrophilic in nature to avoid hydrophobic interaction and electrostatic interaction with mucin[20,21].

2. To attain an appropriate concentration of drug in systemic circulation for receiving a required pharmacological response
3. To reduce dose: Hydrophobic drugs normally require high dose regimen to influence therapeutic plasma concentrations after administration
4. In case of orally administered drugs, solubility plays prime rate limiting parameters to attain desired concentration in complete circulation for pharmacological response[21].
5. To overcome non-uniform distribution: Molecules such as essential oils are commonly used in the food industry as a flavoring and preserving agent but non-uniform distribution in food matrices due to hydrophobic nature reduces their effectiveness[22].

Therefore, essential oils are commonly used with an emulsifier to increase its solubility. However, choice of appropriate emulsifier, its composition, and high molecular weight are significant issues in the application[23]. Conversely, surfactant's low molecular weight and emulsifying activity contribute to wide-ranging industrial uses[24,25]. Recently, biosurfactant-sophorolipids (SLs) are under investigation for enhancing the solubility of a drug due to their myriad advantages like high biodegradability, low toxicity, excellent biocompatibility, easy method of synthesis, more stability and digestibility. As mentioned in the earlier chapter, *C. bombicola* is such non-pathogenic yeast which can convert lipophilic fatty acids (specifically C16-C18 fatty acids) to amphiphilic sophorolipid (SL)[26]. But there are few reports available where *C. bombicola* could convert nonconventional substrate to hydrophilic derivative[27,14,28].

Therefore, in the present work as our essential oil components are also highly hydrophobic in nature we first time applied SL synthesis procedure to convert monoterpene aldehydes (hydrophobic components of essential oil) to their hydrophilic derivative. So far there are no reports on the successful use of monoterpene aldehydes in producing glycomonoterpenes having properties similar to that of sophorolipids (with sophorose unit) or glycolipids (single glucose moiety). Thus, to synthesize novel glycomonoterpenes, glucose and monoterpenes aldehydes -citral, citronellal were used as hydrophilic and lipophilic substrate respectively. In the present chapter, method of synthesis and structural characterization of novel glycomonoterpenes (G-citral and G-citron) is mentioned.

2a.2 Materials and methods

2a.2.1 Chemicals:

Glucose was purchased from Qualigens, India. Peptone, malt and yeast extract were purchased from Hi-media, India. Citral, Citronellal and deuterated chloroform (for NMR analysis) were procured from Sigma Aldrich, India. For extraction, ethyl acetate and sodium sulfate was purchased from Merck, India. Pongamia oil (*Millettia pinnata*) for studying oil displacement activity was purchased from an ayurvedic pharmacy.

2a.2.2 Bacterial strains and culture conditions

Synthesis of glycomonoterpenes (G-citral, synthesized from monoterpene citral, and G-citron, synthesized from monoterpene citronellal) was done using *Candida bombicola* ATCC 22214, as described below.

2a.2.3 Synthesis of glycomonoterpenes from monoterpenes

a) Fermentation conditions

Given that, sophorolipids can be synthesized by two methods, growth-associated and resting cell method. In the present study, both methods of synthesis were tried, and yield of glyco-monoterpenes were compared. In case of resting cell method, *C. bombicola* ATCC 22214 was grown in 10 ml malt extract, glucose, yeast extract and peptone (MGYP) broth for 24 h at 28 °C and 180 rpm and then was transferred to 90 ml MGYP broth and incubated for another 48 h. Cells were collected by centrifugation at 5000 rpm for 10 min and further transferred to 100 ml production medium of 10 % (w/v) glucose. Monoterpene aldehydes, citral and citronellal were added in three different concentrations to optimize concentration of lipophilic substrate (0.3 %v/v, 0.4 %v/v, 0.5 %v/v 0.6 %v/v) to the production medium as lipophilic substrate. Flasks were incubated at 28 °C and 180 rpm for seven days for the synthesis of respective glycomonoterpenes[14]. However, in case of growth associated method, after growing the cells in growth medium for 48 h, in the same medium 10 %(w/v) glucose and different concentrations of lipophilic substrate mentioned in resting cell method were added and incubated at 28 °C and 180 rpm for 7 days for the synthesis.

Table 2a.1 Media composition for sophorolipid production

Media components	Malt extract	Glucose	Yeast extract	Peptone
Concentration(g/L)	3	20	3	5

b) Extraction of glycomonoterpene:

C. bombicola cells from both methods were harvested by centrifugation at 5000 rpm 10 °C for 20 min. This cell mass was reutilized for the glycomonoterpene production again by two methods resting cell and growth associated by using 10 %(w/v) glucose and MGYP media (mentioned in Table 2a.1) with the addition of 8 %(w/v) glucose respectively. Supernatants obtained after centrifugation were extracted thrice with an equal volume of ethyl acetate[29]. Further, sodium sulfate was added to extracted ethyl acetate to remove traces of water and after treatment, was removed by fltration. The product was concentrated by rotary evaporation under vacuum and further purged to remove any traces of solvent to obtain the yellowish viscous products, referred as G-citron and G-citral and were stored at 4 °C. Synthesized glycomonoterpenes (G-citral and G-citron) were then characterized using different analytical techniques.

2a.2.4 Oil displacement activity of synthesized glycomonoterpene

Oil displacement assay was done to confirm the surfactant property of the newly synthesized compounds. For doing assay, in a 100 mm× 15 mm petri plate, 20 ml distilled water was taken and on top of water at the center of petri plate, 500 µl Pongamia oil (*Millettia pinnata*) was added and allowed to form a stable film. Further, on this oil film, 0.5 mg/ml of G-citral and G-citron was added. A clear zone formed after the addition of a glycomonoterpene to oil-water interphase was measured[14].

2a.2.5 Surface tension measurement and CMC estimation:

The surface activities of glycomonoterpenes were measured by direct surface tension measurements. There are different tensiometers with different measuring methods. In this study, a force tensiometer based on Wilhelmy plate method was used for measuring the surface tension of water before and after addition of synthesized glycomonoterpenes (Figure 2a.3). The stock solutions of G-citral and G-citron were prepared in MiliQ water and diluted to the concentration range of 0.0001-0.01 % w/v.

Initially, an instrument was calibrated using MiliQ water and the surface tension was measured after adding different G-citral, G-citron concentrations. In this method when a thin platinum plate was vertically suspended in the G-citral, G-citron solution, surface tension σ observed was as per equation given below.

$$\sigma = \frac{F}{L \times \cos\theta}$$

Where,

F= Force acting on a plate

L= Wetted perimeter of plate = 2t+2w

t = Thickness of plate

w = Width of plate

θ = Contact angle of the plate and the liquid

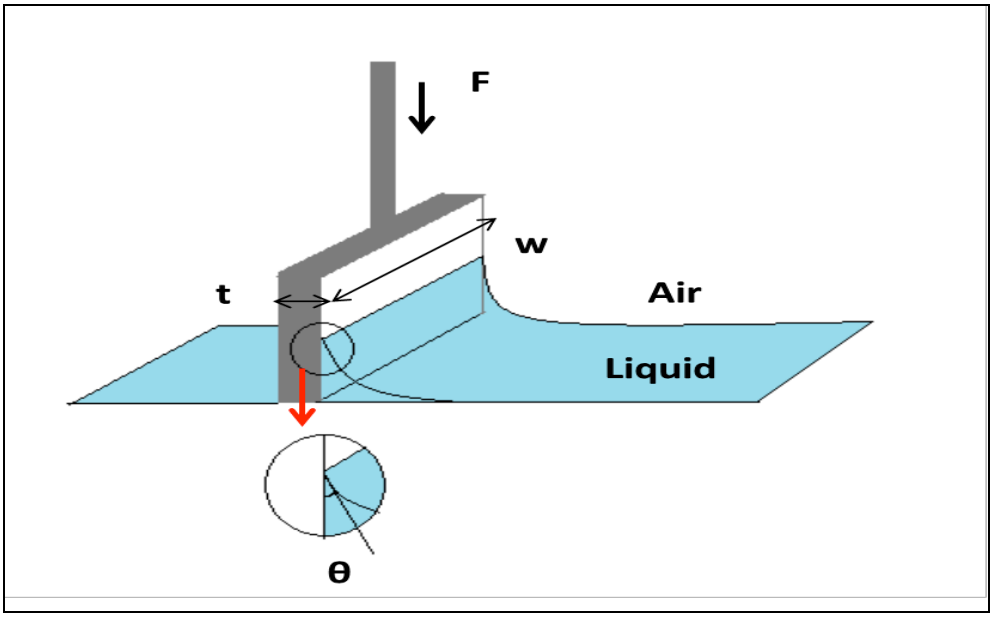


Figure 2a.3 Drawing describing basics of Wilhelmy plate method (adapted from Kruss scientific page)

The critical micelle concentration (CMC) at which surface tension remains relatively constant or changes with a lower slope was determined based on surface tension vs. concentration graph of G-citral and G-citron.

2.2.6 Thin layer chromatography:

Newly synthesized glycomonoterpenes were compared with hydrophobic parent compound using thin layer chromatography (TLC). TLC was performed on commercially available silica gel coated aluminum sheets (Merck Aluminum TLC Silica Gel Plates 60 F 254). The solvent system used was chloroform: methanol (v/v) 97: 3 and 92.5: 7.5 for G-citral and G-citronellal respectively. The bands on the silica gel were visualized by spraying anisaldehyde stain (135 ml absolute ethanol; 5 ml conc. H_2SO_4; 1.5 ml glacial acetic acid; 3.7 ml of P-anisaldehyde) and heating at 140 °C. The band migration pattern obtained with glycomonoterpenes were compared with parent molecules[27].

2.2.7 High-Performance Liquid Chromatography

Parent monoterpene aldehydes-citral, citronellal and newly synthesized glycol monoterpenes were individually analyzed on a Hitachi Chromeline HPLC system using a Thermo Scientific C18 Reversed-Phase HPLC Column 250 × 4.6 mm. A 0–100 % acetonitrile gradient in water over 60 min was used, followed by column re-equilibration for 10 min. A blank run was given between each analysis. The flow rate and column temperature were maintained at 1.0 ml/min and 40 °C respectively, and all 4 compounds were detected at 220 nm.

2.2.8 FTIR of synthesized glycomonoterpenes

FTIR spectroscopic analysis of the synthesized glycomonoterpenes and respective monoterpenes was done using Bruker ATR-FTIR Spectrophotometer over the spectral range of 400– 4000 cm^{-1}. Spectral data obtained was plotted on a graph of transmittance versus wavenumber (cm^{-1})[27].

2.2.9 MALDI-TOF study of G-Citral and G-Citron

G-citral or G-citron in acetonitrile (2 mg/ml) was mixed with α-cyano-4-hydroxycinnamic acid (CHCA) matrix (10 mg/ml) in 1:2, 1:5 ,1:10 ratio and spotted on a matrix assisted laser desorption/ ionization (MALDI) plate. MALDI-MS study was done using the AB SCIEX TOF/TOF 5800 System[30].

2.2.10 NMR of G-citron:

NMR experiments were carried out on a 700 MHz Bruker Avance III HD spectrometer using a 5 mm BBO probe with z-axis gradient. Parent monoterpene aldehydes- citral, citronellal and newly synthesized G-citral, G-citron were separately dissolved in deuterated chloroform ($CDCl_3$) and deuterated methanol (CD_3OD) at a concentration of 30 mg/ml and analyzed to confirm conversion.

2a.3 Results and discussion

2a.3.1 The yield of glycomonoterpenes through resting cell method and growth associated method:

Citral and citronellal 0.5 % v/v gave the highest yield of G-citral and G-citron respectively. Yield-wise there was no significant difference in the resting cell method and growth associated method. Both the synthesis method gave ~ 4 g/l of G-citral /G-citron and ~ 70 % recovery. However, in the case of growth associated method time required for the synthesis of G-citral and G-citron in the subsequent batches is less as compared to resting cell method. Thus growth associated method will help to make the production process more economical.

2a.3.2 Oil displacement activity

The formation of a clear zone on an addition of a test compound to oil-water interphase is the measure of surfactant activity of a compound. G-citral and G-citron showed clear zones with a diameter of 2.7 and 3.2 cm, respectively (Figure 2a.4), whereas parent monoterpenes citral and citronellal showed no oil displacement activity. This clearly confirmed the conversion of monoterpenes to glycomonoterpenes having surfactant activity.

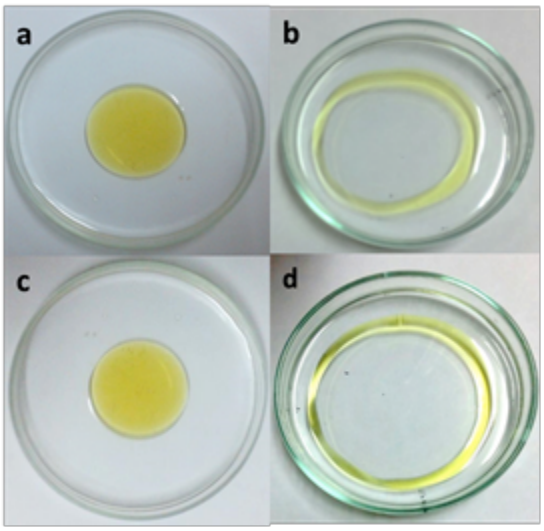


Figure 2a.4 Oil displacement activity of synthesized glycolipid: 1 mg/ml solution of **a.** citronellal, **b.** G-citron, **c.** citral, **d.** G-citral displacing Pongamia oil (*Millettia pinnata*)

2a.3.3 Surface tension and CMC:

We found that G-citron and G-citral reduced the surface tension of distilled water from 71.956 ± 0.5 mN/m to a minimum value of 34.359 ± 0.5 mN/m and 35.254± 0.5 mN/m respectively. From the graph of surface tension vs. concentration, we found out that G-citral and G-citron have CMC value of 80 mg/L and 70 mg/L respectively (Figure 2a.5). This clearly confirmed the conversion of monoterpenes to glycomonoterpenes having surfactant activity.

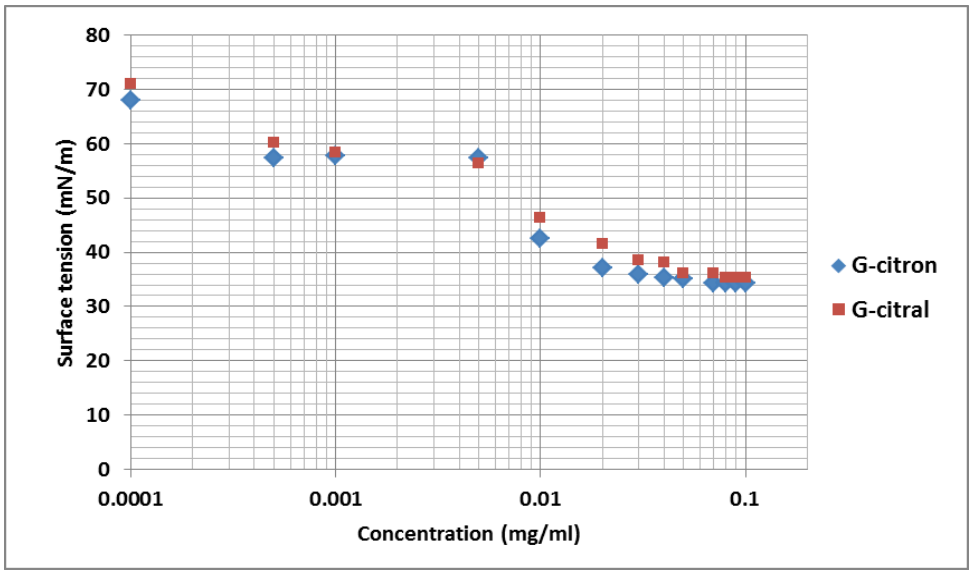


Figure 2a.5 Graphical representation of surface tension reduction with increasing concentration of G-citral and G-citron and measurement of CMC

In comparison to other surfactants, glycomonoterpenes displayed best surface tension lowering ability which is comparable with CMC of previously reported SL-biosurfactant and commercially available surfactant Tomadol 1st series Tomadol 1-5, Tomadol 1-7, Tomadol 1-9, Tomadol 1-73B as well as with Triton X-100 as shown in Table 2a.2

Table 2a.2 Comparative table of surface tension and CMC values of earlier reported biosurfactants and commercially available chemical surfactant

Sr.No.	Surfactant	CMC (mg/l)	Surface tension (mN/m)	Ref.
1.	Palmitic acid sophorolipid	>200	35	[31]
2.	Stearic sophorolipid	35	35	[31]
3.	Oleic acid sophorolipid	140	36	[31]
4.	Linoleic sophorolipid	250	36	[31]
5.	TRITON X-100	150-250	30.6	[32,33]
6.	Tomadol 1 series (ethoxylated alcohol)	100-150	26- 34	[34]

2a.3.4 TLC analysis:

The TLC analysis of glycomonoterpenes (G-citral and G-citron) showed that it is a mixture of the components which resolved on TLC plate corresponding to their respective polarity. G-citral is a mixture of compounds having Rf value 0.93, 0.89, 0.75, 0.67, 0.59, 0.43, 0.25, 0.13 and G-citron is a mixture of compounds with Rf value 0.93, 0.88, 0.84, 0.73, 0.6, 0.53, 0.43, 0.25, 0.12. However, parent monoterpene (Citral /Citronellal) being non-polar showed Rf value 0.86 and 0.91 respectively (Figure 2a.6). Thus, thin layer chromatogram confirmed conversion of Citral and Citronellal to G-citral and G-citron respectively.

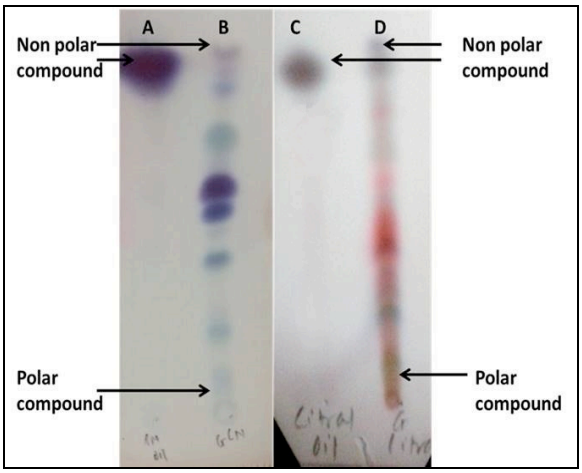


Figure 2a.6 Thin layer chromatography of monoterpenes and synthesized glycomonoterpenes: The solvent system used was chloroform: methanol (v/v) 97: 3 and 92.5: 7.5 for G-Citral and G-citron respectively. The bands on the silica gel were visualized by spraying anisaldehyde stain A: Citronellal B: G-Citron C: Citral D: G-citral.

2a.3.5 HPLC analysis

HPLC analysis was carried out for both the monoterpenes (citral, citronellal) and the resultant glycomonoterpenes (G-citral and G-citron). The gradient elution method was used to analyze the difference in retention time of parent and newly synthesized glycomonoterpenes. Being hydrophobic in nature, citral showed a peak at 36.4 min and citronellal at 41.2 min when the acetonitrile/water ratio was high (Figure. 2a.7). However, G-citral and G-citron eluted in the10–30 -min range when subjected to HPLC under identical conditions. This is also indicative of a polar nature of the compound and is in agreement with TLC results where multiple bands were seen in newly synthesized compounds.

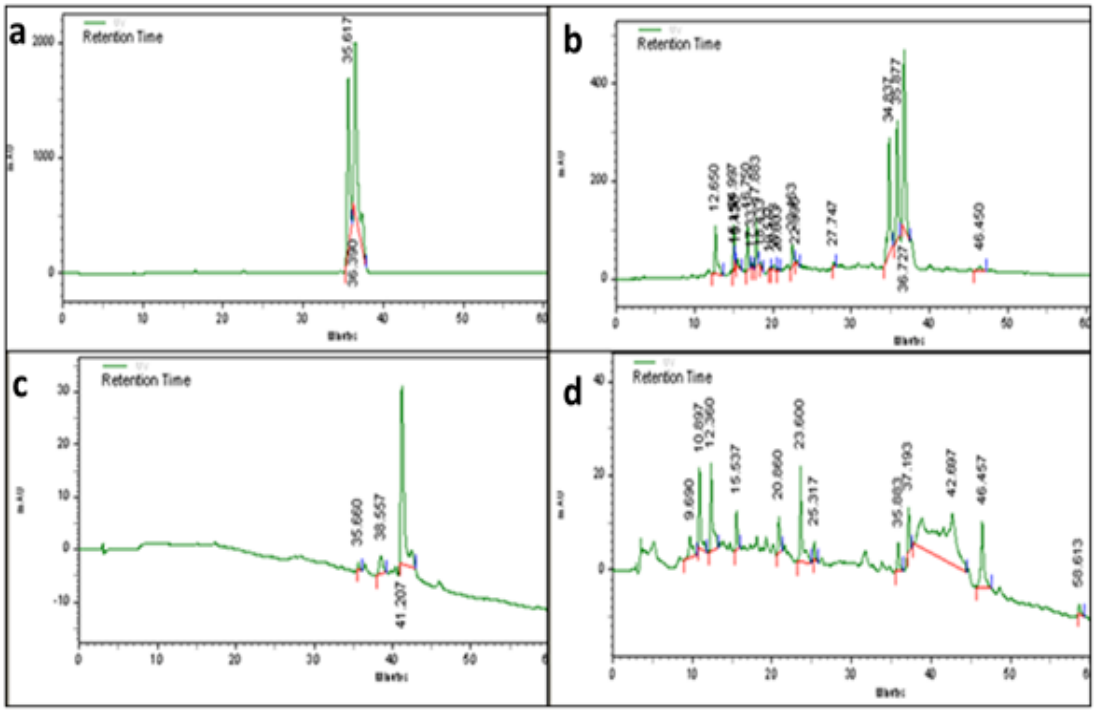


Figure 2a.7 HPLC analysis of monoterpenes citral and citronellal and their respective glycomonoterpenes G-citral and G-citron: HPLC chromatograms of compounds with a gradient elution method, which started with 0 % acetonitrile and 100 % water and ended with 100 % acetonitrile and 0 % water over 60 min, followed by column re-equilibration for 10 min. The flow rate was 1.0 ml/min, and the absorption wavelength was set at 220 nm. a) Citral chromatogram showing retention time 36.39 min b) G-citral spectra showing product elution earlier to 36.39 min c) Citronellal spectra showing retention time 41.21 min d) G-citron chromatogram where peaks earlier to 41.21 min represents a newly synthesized product, i.e., G-citron

2a.3.6 FTIR analysis of synthesized glycomonoterpenes

Analysis of the FTIR spectrum of G-citral and G-citron clearly showed incorporation of the parent moiety into the final product. The spectra clearly showed peaks corresponding to methyl (1379 cm^{-1}) and methylene groups (1452 cm^{-1}) from citral and citronellal. The 3063–3640 cm^{-1} region corresponding to the O–H stretch frequency in the glucose moiety was also observed in the G-citral and G-citron spectra, with the asymmetrical and symmetrical stretch modes of the methylene (CH2) groups of glucose and sophorose occurring at 2857 cm^{-1}. Presence of a C=O

stretch of a saturated aliphatic cyclic six-membered ring of glucose (1720 cm−1) was also evident in the spectra. The carbonyl stretch C=O of saturated aliphatic aldehydes (citronellal) appears at 1729 cm^{-1}, and the carbonyl stretch of the unsaturated aldehyde (citral) showed a shift to a lower wavenumber, 1674 cm^{-1} (Figure. 2a.8). A similar FTIR spectrum has been observed for glycomonoterpene alcohol with an additional peak at 1060 cm^{-1} for C–O stretching from primary alcohols, which was absent in the glycomonoterpenes in the present study[30].

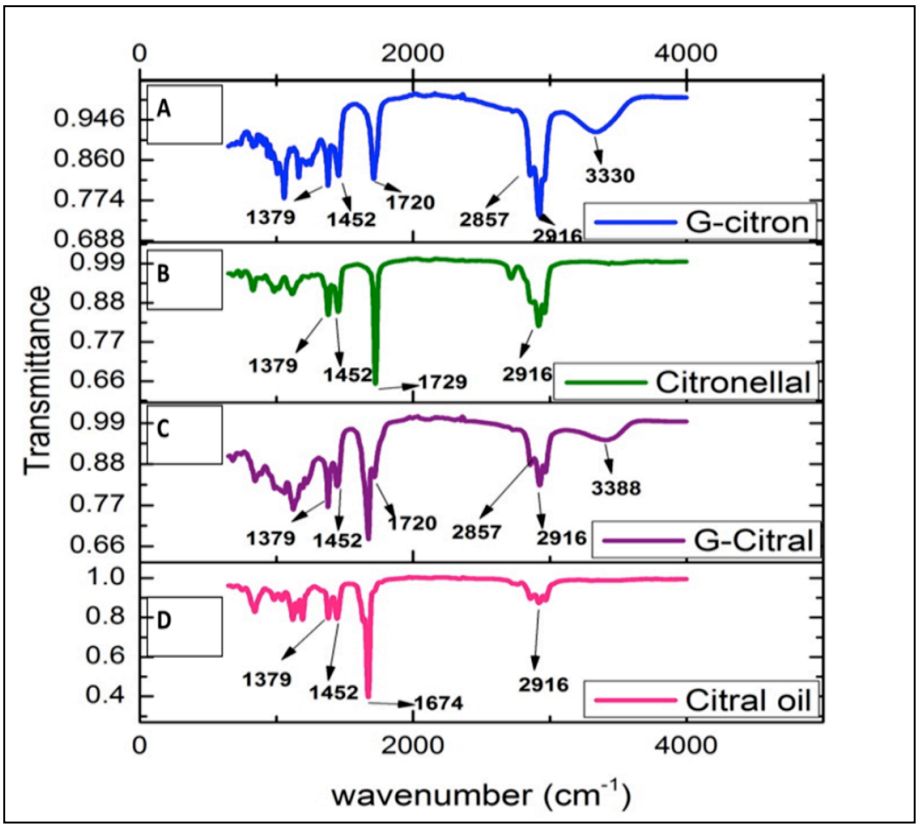


Figure 2a.8 FTIR spectroscopic analysis of parent monoterpenes and synthesized glyco-monoterpenes**: A**. **G-citron:** peak at 1379 cm^{-1} corresponding to methyl groups indicated presence of citronellal in G-citron. A peak at 1452 cm^{-1} represents the presence of the methylene group. The 3063–3640 cm^{-1} region in spectra corresponds to the O–H stretch frequency in the glucose. The asymmetrical and symmetrical stretch modes of methylene (CH_2) groups of glucose and sophorose occur at 2857 cm^{-1}. Presence of C=O stretch of saturated aliphatic cyclic six-membered ring of glucose (1720 cm^{-1}) was also evident in the spectrum of G-citron. **B. Citronellal:** the carbonyl stretch C=O of saturated aliphatic aldehydes (citronellal) appears at 1729

cm−1. **C. G-citral:** peak at 1379 cm^{-1} corresponding to methyl groups indicated the presence of citral in G-citral. Peak at 1452 cm^{-1} represents the presence of methylene group in G-citral. The 3063–3640 cm^{-1} region in spectra corresponds to the O–H stretch frequency in the glucose. The asymmetrical and symmetrical stretch modes of methylene (CH_2) groups of glucose and sophorose occur at 2857 cm^{-1} in G-citral spectrum. Presence of a C=O stretch of a saturated aliphatic cyclic six-membered ring of glucose (1720 cm^{-1}) was also evident in the spectrum of G-citral. **D. Citral:** carbonyl stretch of the unsaturated aldehyde (citral) showed a shift to a lower wavenumber, 1674 cm^{-1}

2a.3.7 MALDI-TOF analysis of G-Citral and G-Citron

Structure of G-citral and G-citron were predicted based on SL synthesis[26] and their molecular weights were calculated (Figure 2a.9) and then compared with MALDI data to verify the structures. In the case of G-citral, two major compounds having m/z 454.4023 $[M1 + 2Na + NH_4 + H]^+$ and 370.4219 $[M2 + Na + H]^+$ were observed (Figure 2a.10 a, b). The molecular weight of these compounds is well correlated with the acidic form of glycolipids having a monoacetylated glucose head group (M1 =389.33) and a glucose head group without acetylation (M2 = 346.2296) (Figure 2a.10 a, b, Table 2a.3). However, in the case of G-citron, three major compounds having m/z 510.55, 438.55, and 348.42 were observed. Theoretical calculations suggest that m/z 510.55 $[M3 + NH_4]^+$ could correspond to the lactonic form of glycolipid possessing a sophorose head group without acetylation where M3 = 492.43 (Figure. 2a.10 c), and the compound showing a peak at 438.55 $[M4 + 2Na + NH_4 + H]^+$ could correspond to the lactonic form of glycolipid with a monoacetylated glucose head group where M4 = 373.47 (Figure 2a.10 c). On the other hand, the peak at m/z 348.42 $[M5+ NH_4]^+$ could be the lactonic form of glycolipid having a glucose head group without acetylation where M5 = 330.39 (Figure 2a.10 d). Thus, all major compounds in G-citron were found to possess a lactonic form of glycolipid (Table 2a.3). The structures obtained in the present study are comparable with earlier reports that glycomonoterpene alcohols can be a mixture of compounds possessing either sophorose or glucose as head group[30].

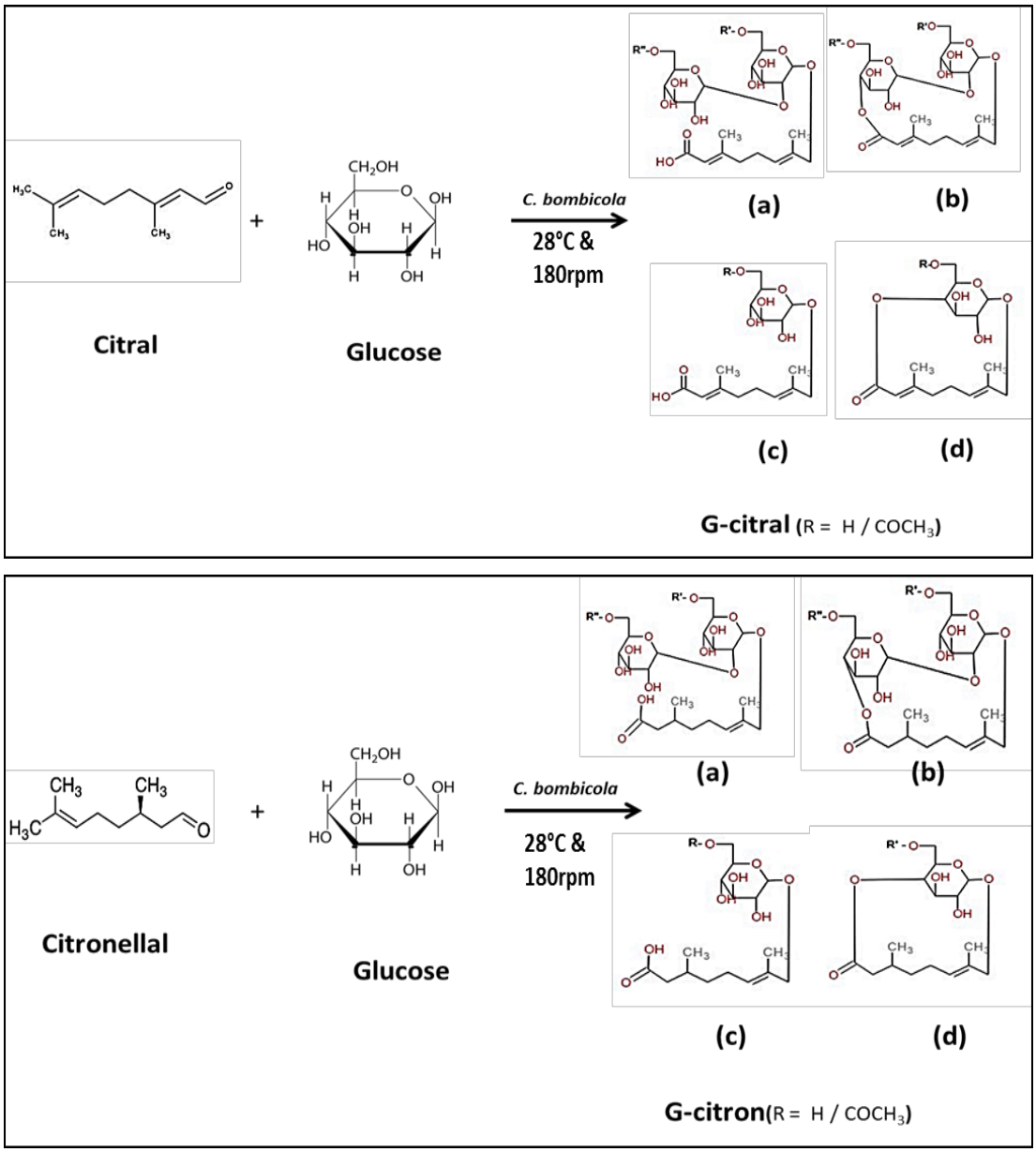


Figure2a.9: Predicted structures of G-citral and G-citron

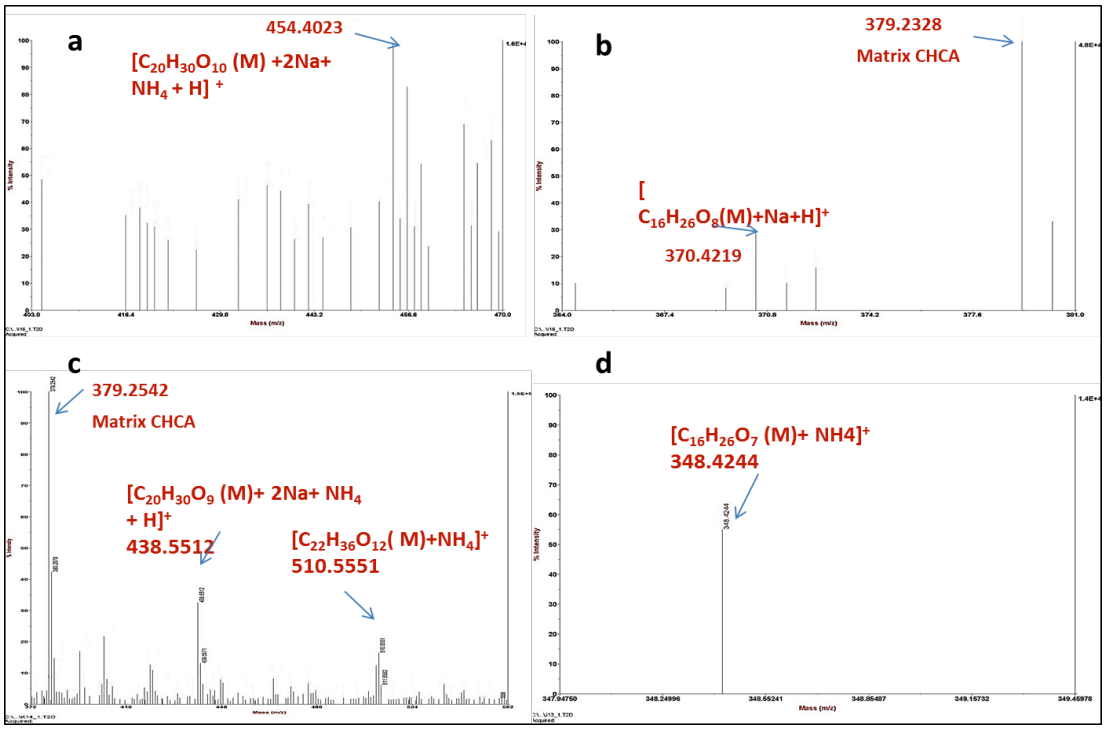


Figure 2a.10 MALDI-TOF analysis of G-citral and G-citron: **a** m/z of G-citral [M + 2Na + NH_4 + H]$^+$ where M=389.33. **b** m/z of G-citral [M + Na + H]$^+$ where M= 346.23. **c** m/z of G-citron [M + 2Na + NH_4 +H]$^+$ and [M + NH_4]$^+$ where M = 373.47 and 492.43, respectively. **d** m/z of G-citron [M + NH_4] $^+$ where M = 330.39

Table 2a.3 Verified forms of synthesized product based on MALDI results:

No.	Compound (m/z)	Form	Structure of synthesized product	Elemental composition	Calculated mass (M)
1	**G-citral**				
	454.4023 [M+2Na+NH_4+H]$^+$	Acidic product	Glucose head group + mono - acetylation	$C_{18}H_{29}O_9$	389.33
	370.4219 [M+Na+H]$^+$	Acidic product	Glucose head group +no acetylation	$C_{16}H_{26}O_8$	346.2296
2	**G-citron**				
	510.5551 [M+NH_4]$^+$	Lactonic product	Sophorose head Group (No acetylation)	$C_{22}H_{36}O_{12}$	492.432
	438.5512 [(M+ 2Na+ NH_4 +H]$^+$	Lactonic product	Glucose head group + mon-acetylation	$C_{18}H_{29}O_8$	373.47
	348.4244 [M+ NH4]$^+$	Lactonic product	Glucose head group (No acetylation	$C_{16}H_{26}O_7$	330.39

2a.3.8 NMR analysis of G-citral and G-citron:

As compared to deuterated methanol (CD_3OD), compounds dissolved in deuterated chloroform ($CDCl_3$) displayed all the signature peaks of compounds which is shown in Figure 2a.11. G-citron showed all characteristic signals from both the sugar rings and the monoterpene aldehydes. In the citronellal spectra H1, H4, H7, H8, H9 were observed which are signature peaks of citronellal and H1 corresponding to aldehyde group proton however in the G-citron sample spectra H4, H7, H8, H9 were observed with similar diffusion coefficients but H1 proton was missing so it can be assumed that there is direct incorporation of citronellal and any change must have happened in the aldehyde part of the molecule (Figure 2a.11 and Figure 2a.12).

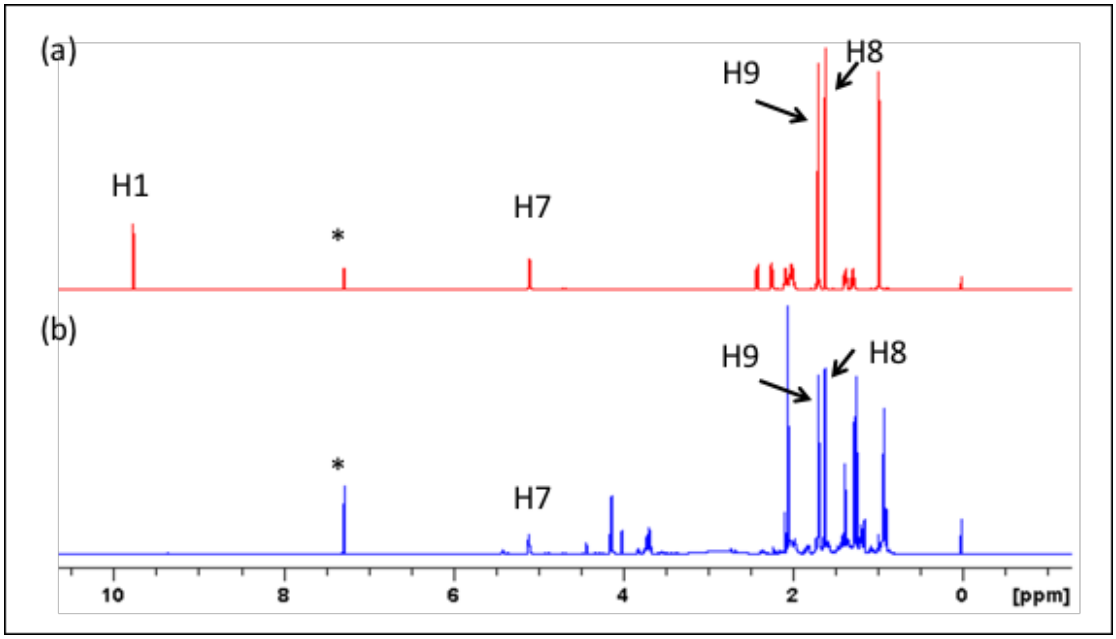


Figure 2a.11: ^1H NMR spectra of a) Citronellal b) G-citron obtained on a 700 MHz spectrometer at 298 K. Signal assignments are indicated for citronellal and G-citron, with residual solvent signal marked by asterisks.

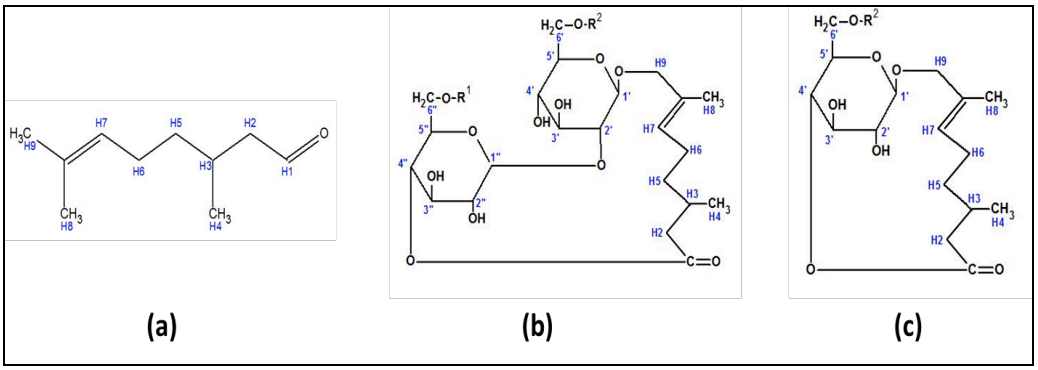


Figure 2a.12: Representation of structures of citronellal and G-citron (lactonic form) molecules with the labeling of H atoms: (a) citronellal (b) Lactonic G-citron (glycomonoterpene) with sophorose unit (c) Lactonic G-citron (glycomonoterpene) with glucose moiety

Similar results were also observed with G-citral and citral. G-citral showed all representative signals from both the sugar rings and the monoterpene aldehydes. In the citral spectra H1, H2, H6, H7, H8 were observed which are signature peaks of citral where H1 corresponds to aldehyde group proton (Figure 2a.13, 2a.14). However, in the G-citral sample spectra except H1 all other signature peaks (H2, H6, H7, H8) were observed with similar diffusion coefficients. Therefore, it can be assumed that there is direct incorporation of citral and any modification must have occurred in the aldehyde group of the citral (Figure 2a.13, 2a.14).

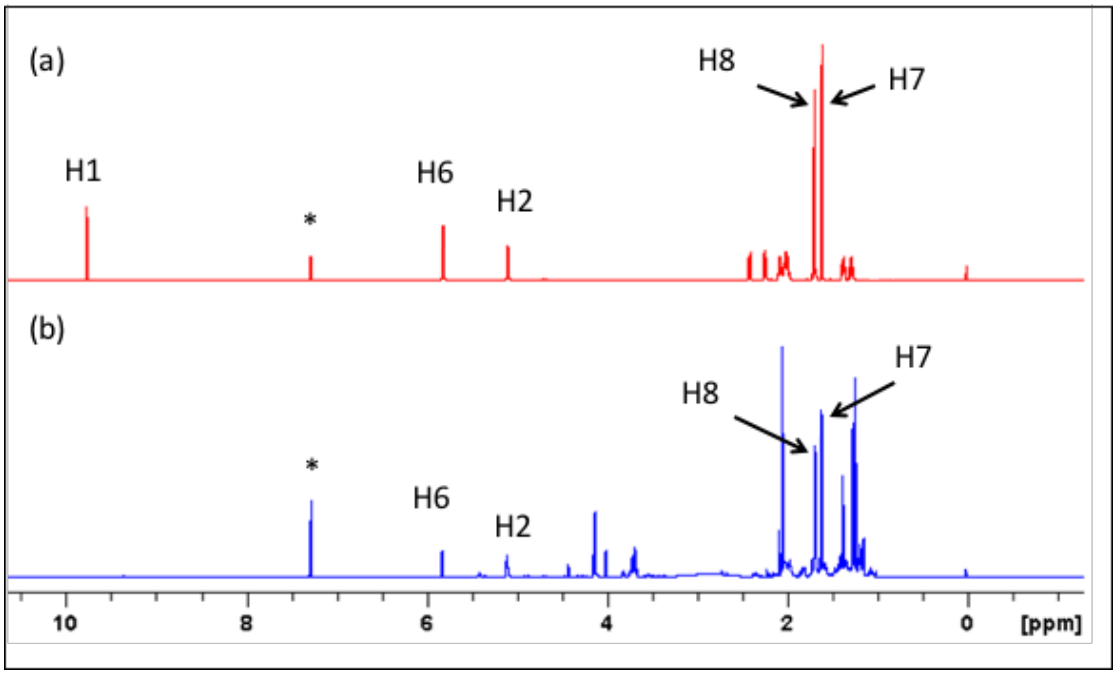


Figure 2a.13: ^{1}H NMR spectra of a) citral b) G-citral obtained on a 700 MHz spectrometer at 298 K. Signal assignments are indicated for citral and G-citral with residual solvent signal marked by asterisks.

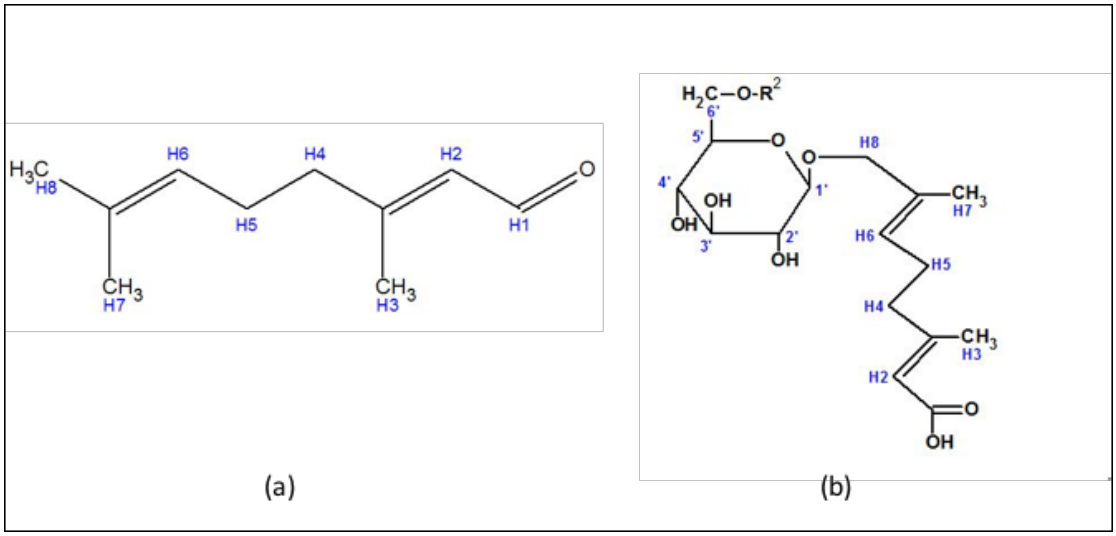


Figure 2a.14: Representation of structures of citral and G-citral (acidic form) molecules with the labeling of H atoms: (a) citral (b) Acidic G-citral (glycomonoterpene) with glucose moiety

2a.4 Conclusion:

The present chapter highlights the successful conversion of the major hydrophobic components of lemongrass oil, citral and citronellal to G-citral and G-

citron respectively. In this study, we showed that *C. bombicola* was able to convert non-conventional substrate monoterpene aldehyde (citral and citronellal) to glycomonoterpenes (G-citral and G-citron) even in the absence of an inducer. Considering time required for the synthesis in the subsequent batches, growth-associated method found to be more advantageous as compared to resting cell method. Oil displacement assay and CMC measurement proved surfactant nature of G-citral and G-citron giving ~70 mg/L CMC value which is lower than commercially available Triton 100X and Tomadol 1st series surfactant. Lower CMC value of G-citral and G-citron is an indication of improved solubility thereby enhanced oral bioavailability of glycomonoterpene based drugs. TLC, HPLC, FTIR, NMR confirmed successful conversion and direct incorporation monoterpene aldehydes to glycomonoterpenes by modification at aldehyde group. The product synthesized was characterized to confirm conversion and for predicting structure. MALDI analysis confirmed predicted structure and showed that G-citron synthesized using citronellal contains three major lactonic forms with molecular weight 492.43, 473.47, and 330.39 Da whereas G-citral synthesized using citral has an acidic form with molecular weight 389.33 and 346.23 Da.

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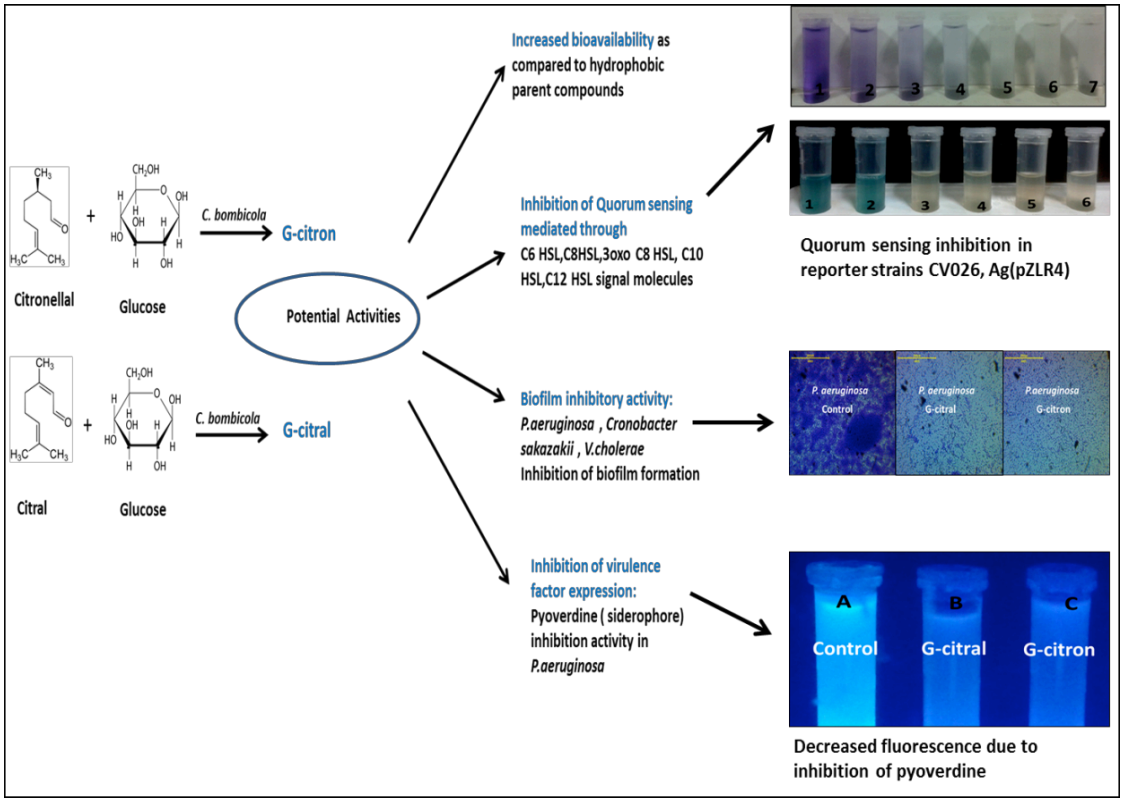
Chapter 2b

Exploring biological activity of glycomonoterpenes (G -citral, and G-citron) as quorum sensing inhibitory molecules.



Recently, an alarming increase in the development of multidrug-resistant bacteria has become a major threat. Therefore, newer drug targets with a significantly lower risk of development of resistance are needed. Targeting quorum sensing in bacteria does not affect growth instead inhibit pathogenesis mediated through quorum sensing thus this strategy is less likely to develop resistance in the future. Quorum sensing initiates the formation of biofilms and offers numerous advantages to bacteria by protecting them from antibiotics as well as host immune response. Essential oils (EOs) have always got an important place in traditional medicine, and recently few reports are also available on quorum sensing inhibition. However, hydrophobic nature and complexity of essential oils limit their medical application. Therefore, in the

present study, we have used the hydrophilic derivative of major components of lemongrass essential oil-G-citron, G-citral to assess their quorum sensing inhibitory activity after conversion and we found that the glycomonoterpenes (G-citral and G-citron) were able to individually inhibit QS, mediated through various medium-chain and long-chain N-acyl homoserine lactones (AHLs). These new compounds are interesting additions to the known range of quorum sensing inhibitors (QSIs) and could be further explored for potential clinical applications.

2b.1 Introduction

In the last few decades, numerous bacterial strains have developed resistance to various commonly used antibiotics, due to genetic, social, and environmental factors. This has led to the predominance of multiple-drug-resistant (MDR) and extremely drug-resistant (XDR) bacteria[1]. Global surveillance report of antimicrobial resistance by WHO in 2014 stated that that the world has entered into a post-antibiotic era in which infections which were easily curable for decades may once again cause problem[2]. Biofilm forming ability of the bacteria can be considered a major reason behind the problem of antibiotic resistance.

Earlier it was assumed that bacteria are present in the planktonic state but research findings from last few decades revealed multicellular existence of bacteria. Biofilm is a unique multicellular structure where bacteria are embedded in the self-secreted extracellular polysaccharide matrix and proteinaceous material, which protects them from environmental stresses, antimicrobial agents, and the host immune system[3]. Inside biofilm bacteria are 1000 times more resistant to antibiotics as compared to their planktonic counterparts[4].

The National Institute of Health (NIH) found that around 65 % and 80 % of all microbial and chronic infections respectively are associated with biofilm formation. Bacteria form a biofilm on both biotic and abiotic surfaces and cause both, device- and non-device-associated infections. Device-associated biofilm infections are commonly caused due to bacterial biofilms on or within medical devices such as contact lenses, central venous catheters, mechanical heart valves, peritoneal dialysis catheters, prosthetic joints, pacemakers, urinary catheters, and voice prostheses. However, non-device associated infections include periodontitis, severe burn associated infections, chronic lung infections in patients with cystic fibrosis, Osteomyelitis, and nosocomial pneumonia[5].

The formation and maintenance of biofilms are governed by bacterial quorum sensing (QS) dependent gene expression[6]. Quorum sensing is basically the ability of bacteria to communicate with other surrounding bacteria. It is a density-dependent phenomenon which enables bacteria to do quorum sensing and eventually provides protection from host immune response and other harsh environmental conditions which are otherwise deleterious. Quorum sensing is mediated through small, easily diffusible signal molecules usually known as autoinducers which allow bacteria to sense their population density and to control the gene expression in response to the cell density [7]. QS is known to regulate a range of bacterial functions such as bioluminescence, nitrogen fixation, expression of virulence factors, biofilm formation, and swarming motility which has been shown to contribute to bacterial pathogenesis[8]. Targeting QS pathways in bacteria could be an effective strategy to circumvent the problem of multiple drug resistance in bacteria. Quorum sensing inhibitors are molecules that can incapacitate pathogenic bacteria by exclusively interrupting the QS mechanism without putting selective pressure on their growth[6,9] and this property make them different from antibiotics[6,9,10]. QS in bacteria can be hindered by several ways which ultimately can be used to inhibit pathogenicity[6,9]. Briefly, by (1) Inhibition of signal binding to LuxR-family receptor, (2) Inhibition of signal synthesis, (3) Degradation of signals, (4)Trapping of signals, and (5) Suppression of LuxI –family synthase and LuxR-family receptor synthesis and their activities[1,6,11]

There are four major quorum sensing systems Autoinducer type 1 (AI-1) QS system (Gram-negative bacteria), Autoinducer type-2 (AI-2) QS system (Gram-positive and Gram-negative), Autoinducer system -3 (AI-3) QS system (Enteric Gram-negative bacteria) and Quorum sensing system in Gram-positive bacteria[1]. Gram-negative bacteria are more dangerous and pose a significant scientific challenge as a pathogenic organism because of their unique cell wall features. Gram-negative bacteremia has been linked with severe sepsis, and incidences are higher among patients in the adult intensive care unit. As per one study, it was observed that C-reactive protein and IL-6 levels were significantly higher in Gram-negative bacteremia than in Gram-positive bacteremia. These findings propose an early onset of immunopathophysiologic behavior of sepsis in patients with Gram-negative bacteremia[12]. Conventional antibiotics such as erythromycin, vancomycin, linezolid, rifampicin, etc. are ineffective against Gram-negative bacteria, particularly in their biofilms[13].

Gram-negative quorum sensing system is majorly mediated through autoinducer type-1 signal molecule called as N-acyl homoserine lactone (AHL). This signal molecule possesses homoserine lactone ring linked to either a short (C < 8) or a long (C ≥ 8) acyl chain through an amide bond. The short-chain AHL passively diffuses through bacterial membranes, and the long-chain AHL signals require active transportation mechanisms for their efflux[8]. Lux I gene is responsible for the expression of AHL synthase whereas LuxR encodes the receptor for AHL. AHLs are synthesized by enzyme LuxI with two substrates, fatty acyl substrates presented by Acyl Carrier Protein (ACP) or Co-enzyme-A (CoA) and S-adenosyl methionine (SAM). Attainment of the threshold concentration of AHL outside the cell leads to recognition of AHL by N-binding domain of LuxR associated with the cytoplasmic membrane. Upon activation by AHL, LuxR dissociates from the membrane and change its conformation to form a dimer. C-terminal domain of AHL-LuxR dimer binds to Lux-box and autoinduces QS system by transcription of luxI gene. AHL-LuxR dimer also initiates expression of genes associated with QS mediated phenotypes (Figure 1.3-Chapter 1) [11]

Recently, essential oils have been reported as quorum sensing inhibitors (Chapter 1 Table 1.5) however hydrophobic nature, antibacterial activity, and complexity impose major problem in exploring their real potential in biomedical applications in terms of quorum sensing inhibition. So rational behind this chapter was to assess quorum sensing inhibitory activity of novel hydrophilic derivative of two major components of essential oil G-citral and G-citron (synthesis mentioned in Chapter 2a) after conversion. So to evaluate the inhibition of quorum sensing mediated through the short chain and long chain AHL molecule two reporter strains *Chromobacterium violaceium* - CV026 and *Agrobacterium tumefaciens* -Ag(pZLR4) respectively were used. The effect of G-citral and G-citron on quorum sensing mediated phenotypes (biofilm formation and virulence factor production) was also studied.

2b.2 Materials and methods

2b.2.1 Bacterial strains and culture conditions:

Biosensor strains used for detection of QSI activity include *C. violaceum* CV026 (a kind gift from Dr. Paul Williams, University of Nottingham), a mini-tn5 mutant of wild-type *C. violaceum*, and *A. tumefaciens* NTL4(pZLR4) (kindly given by Dr. Stephen Farrand, University of Illinois, USA). *A. tumef*aciens strain NTL4 is a

non-pathogenic derivative of strain C58. Plasmid pZLR4 contains two key units, the traR gene, and a traCDG::lacZ fusion.CV026 was grown in Luria- Bertani (LB) medium supplemented with 100 µg/ml ampicillin and 30 µg/ml kanamycin at 28 °C. *A. tumefaciens* NTL4(pZLR4) was grown in nutrient broth with 30 µg/ml gentamicin at 28 °C. To study the antibiofilm activity of glycomonoterpenes, *Pseudomonas aeruginosa* NCIM 5029, *Cronobacter sakazakii* ATCC 12868 and *Vibrio cholerae* MTCC 0139 were used. Pyoverdine inhibition due to glycomonoterpenes was studied against *P. aeruginosa* NCIM 5029 cultured in Kings' B medium at 37 °C

2b.2.2 *C. violaceum* CV026-based violacein inhibition assay:

C. violaceum is a Gram-negative bacterium which produces violacein pigment in response to quorum sensing regulated gene expression. However, CV026 is deficient in autoinducer synthase and requires an exogenous addition of AHL to produce violacein. Therefore this strain is a useful tool for the biological assay of screening QS inhibitors[14]. CV026-based violacein inhibition assay is used specifically to check inhibition mediated through short chain AHL[15]. In the current study, violacein production was determined to study the potential of glycomonoterpenes to inhibit quorum sensing mediated by N-hexanoyl-L-homoserine lactone (C6-HSL) and N-octanoyl-L-homoserine lactone (C8-HSL). CV026 (0.2 % inoculum from overnight culture) was inoculated in 2 ml LB broth containing 100 µg/ml ampicillin and 30 µg/ml kanamycin, along with 0.1 µl of C6-HSL (25 mM stock) and the test sample (G-citral or G-citron). Six concentrations of test samples in water were used (0.05, 0.1, 0.25, 0.5, 0.75, and 1 mg/ml). After overnight incubation at 28 °C and 180 rpm, violacein pigment produced was extracted with dimethyl sulfoxide (DMSO) and measured spectrophotometrically at 580 nm. The effect of the glycomonoterpenes on C8-HSL-mediated quorum sensing was also studied using the same method [16]. Violacein inhibition (%) was calculated in comparison with the control using the following formula [17]:

$$\text{Violacein inhibition } (\%) = \frac{(Control\ OD_{580\ nm} - Test\ OD_{580\ nm}) \times 100}{Control\ OD_{580\ nm}}$$

2b.2.3 *A. tumefaciens* NTL4 (pZLR4)-based pigment inhibition assay:

A. tumefaciens NTL4(pZLR4) is a TraR-based detector strain which lacks Ti plasmid and traI thus it is non-pathogenic and does not produce acyl homoserine

lactones endogenously. Plasmid pZLR4 has a copy of traR and fusion of lacZ to traG which is expressed from TraR dependent promoter. In the absence of exogenous signal, strain does not produce sufficient amount of β-galactosidase and produce white colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) containing media. However, when exposed to exogenous signal strain produces blue colonies on X-gal containing medium due to a production of β-galctosidase[18]. *A. tumefaciens NTL4(pZLR4)*-based pigment inhibition assay is used to check the inhibition of quorum sensing mediated through long-chain AHL[15]. In our study, we have executed this assay to check the inhibition of quorum sensing mediated through 3-oxo-C8-HSL (N-3 oxo-octanoyl- L-homoserine lactone), C10-HSL (N-decanoyl-L-homoserine lactone), and C12-HSL (N-dodecanoyl-L-homoserine lactone). The reporter system in this strain is based on β-galactosidase activity and produces a blue pigment in the presence of externally provided X-gal and a signal molecule. *A. tumefaciens NTL4 (pZLR4)* was inoculated (10 % inoculum from overnight culture) in 1 ml NB containing 30 µg/ml gentamicin, the respective signal molecule (0.25 µg/ml), X-gal (60 µg/ml), and a test compound. Five different concentrations of sample were tested (0.1, 0.25, 0.5, 0.75, and 1 mg/ml). A tube without any test compound was used as a control. Incubation was done overnight at 28 °C and 180 rpm. Quantification method of *Agrobacterium tumefaciens* NTL4(pZLR4) reporter strain based quorum sensing assay has not been reported previously. Pigment obtained after X-gal degradation is 5, 5'-dibromo-4, 4'-dichloro-indigo and there are reports on solubility of indigo products in DMSO. Therefore, in our study the quantification of 5,5'-dibromo-4,4'-dichloro-indigo was done by dissolving product in DMSO and quantified by taking O.D. at 630 nm (λ_{max} of pigment dissolved in DMSO)[19].

$$\text{Pigment inhibition } (\%) = \frac{(Control\ OD_{630\ nm} - Test\ OD_{630\ nm}) \times 100}{Control\ OD_{630\ nm}}$$

2b.2.4 Antibiofilm assay of glycomonoterpenes:

The antibiofilm activity of the glycomonoterpenes was evaluated using *P. aeruginosa*, *C. sakazakii* and *V. cholerae* as test organisms. Initially, 10 µl of overnight grown cultures of *P. aeruginosa C. sakazakii* and *V. cholerae*, were subcultured to petri plates (30 mm diameter) containing 2 ml LB media and a sterile coverslip. Six different concentrations (0.25, 0.5, 0.75, 1, 1.5, 2 mg/ml) of G-citral or G-citron were added to the plates. After 24 h at 37 °C, biofilms formed in control and

treated plates were visualized by staining with crystal violet. The stained biofilm was then visualized under a light microscope at ×400 magnification[20,21]. The antibiofilm activity of glycomonoterpenes was also evaluated quantitatively using *P. aeruginosa*, *C. sakazakii* and *V. cholerae*. For quantitative evaluation of antibiofilm activity, initially biofilm was stained with crystal violet, and then crystal violet was extracted using 30 % acetic acid. O.D. was measured at 580 nm.

2b.2.5 Pyoverdine inhibition assay:

Quorum sensing in bacteria also governs virulence factor production. *P. aeruginosa* produces pyoverdine which is a virulence factor involved in chronic infection. For assessing the inhibition of pyoverdine production by *P. aeruginosa* in the presence of glycomonoterpenes,10 µl overnight grown culture of *P. aeruginosa* was transferred to 2 ml of King's medium B and incubated in the presence 1 mg G-citral or G-citron at 35 °C. Pyoverdine which is produced after 12 h was measured by recording the fluorescence of the cell-free supernatant, with excitation at 405 nm and emission at 465 nm[22].

2b.2.6 Statistical analysis

Experiments were performed in triplicates, and the data obtained from the experiments were given as mean values. To determine if two sets of data (control and treated) are significantly different from each other, Student's t test was used[21]. Graph pad prism 5 software was used to do statistical analysis.

2b.3 Results and discussion

2b.3.1 *C. violaceum* CV026-based violacein inhibition:

Quorum sensing inhibitory activity of G-citral and G-citron was studied quantitatively using the *C. violaceum* CV026 reporter strain. QSI activity was checked against C6 HSL and C8 HSL signal molecules. Citral and citronellal showed growth inhibition at 0.5 mg/ml (Figure 2b.1 E). However, G-citron showed ~100 % pigment inhibition at the same concentration when the C6 HSL signal molecule was added externally in the CV026 culture (Figure 2b.1 B) and at even lower concentration (0.25 mg/ml) when C8 HSL was used (Figure 2b.1 D). G-citral completely inhibited pigment production at 0.5 mg/ml concentration, with both C6

HSL and C8 HSL (Figure 2b.1 A, C). The reduction in pigment production was observed solely as a result of quorum sensing inhibition and not due to growth inhibition, as evidenced by the absence of any change in OD_{600nm} (Figure. 2b.1E). This result is consistent with an earlier report where they have quantitatively shown that monoterpene alcohols, linalool, and terpineol inhibits quorum sensing mediated through short chain AHL signal molecule[20].

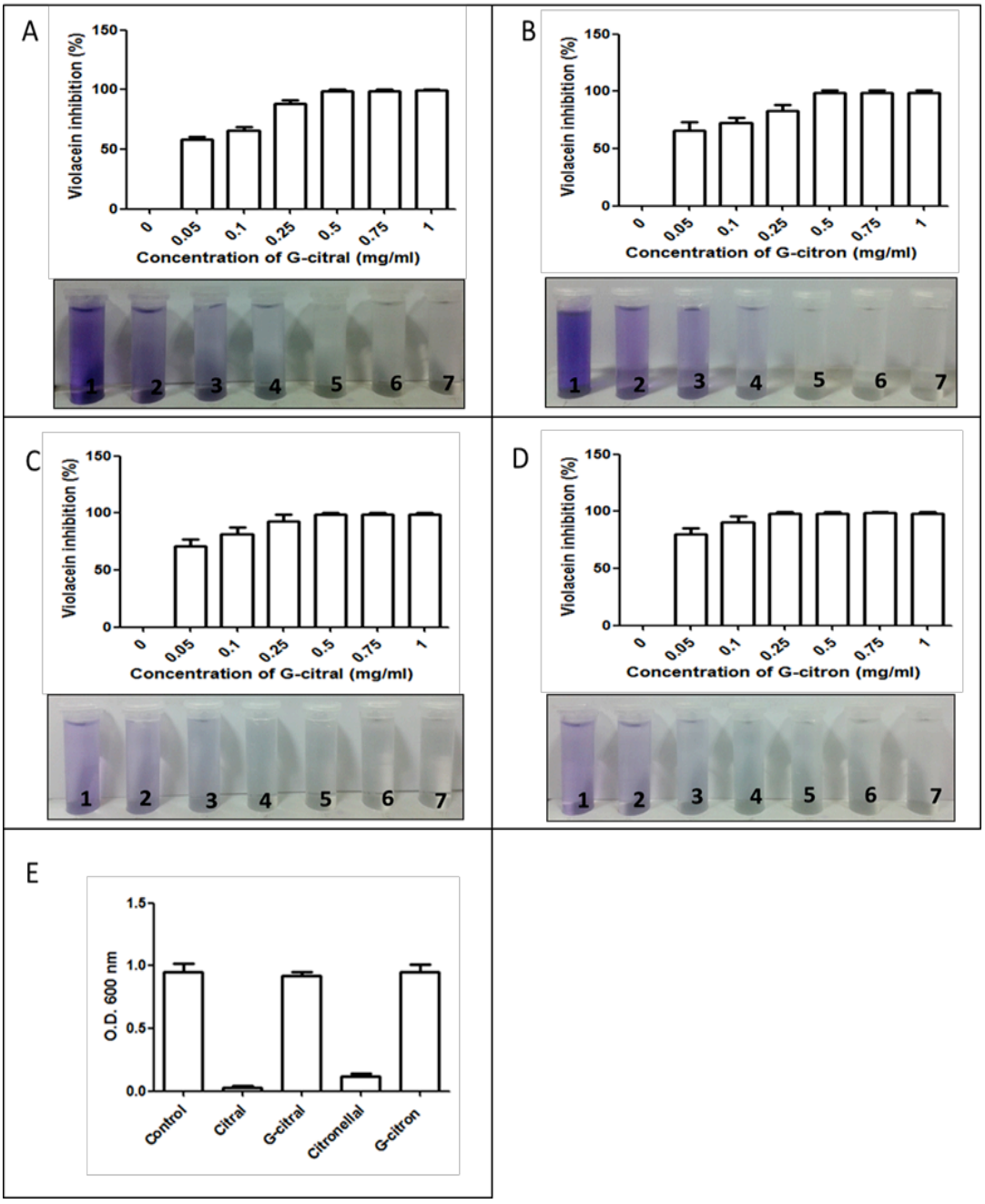


Figure 2b.1: Quantitative analysis of violacein inhibition in CV026 by G-citral and G-citron and effect of optimum concentration on growth of CV026: data is represented as the percentage of violacein inhibition. Mean values of triplicate

independent experiments and SE are shown A and B: Violacein inhibition in the presence of C6 HSL as external signal molecule due to G-citral and G-citron respectively, significant at $p < 0.005$. C and D: Violacein inhibition in the presence of C8 HSL as external signal molecule due to G-citral and G-citron respectively, significant at $p < 0.005$. E: Effect of 0.5 mg/ml concentration of G-citron, G-citral, citronellal, and citral on growth of CV026 (1, 2, 3, 4, 5, 6, 7 corresponds to 0 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.25 mg/ml,0.5 mg/ml, 0.75 mg/ml, 1 mg/ml concentration of test compound)

2b.3.2 *A. tumefaciens* NTL4 (pZLR4)-based pigment inhibition:

A. tumefaciens NTL4 (pZLR4)-based assay was done to assess the inhibition of quorum sensing activity governed by long-chain signal molecules, viz., 3-oxo-C8-HSL, C10-HSL, and C12-HSL. The parent monoterpenes (1 mg/ml) showed growth inhibition whereas the same concentration of G-citral and G-citron did not affect bacterial growth (Figure 2b.2). G-citral (0.75 mg/ml) showed complete inhibition of pigment production when 3-oxo-C8-HSL was added as external signal molecule (Figure 2b.3 A). In the case of C10-HSL and C12-HSL, 0.75 mg/ml concentration of G-citral was enough to achieve more than 90 % pigment inhibition, but to attain nearly complete pigment inhibition,1 mg/ml was required (Figure 2b.3 C, E). Interestingly, we observed that 0.75 mg/ml concentration of G-citron could achieve more than 95 % pigment inhibition with 3-oxo-C8-HSL as the signal, but a concentration of 1 mg/ml was required for complete inhibition (Figure 2b.3 B). However, 0.75 mg/ml concentration of G-citron was enough to achieve complete pigment inhibition when C10-HSL and C12-HSL signals were used (Figure 2b.3D, F). Many pathogenic bacteria produce long chain AHL molecules e.g. *P. aeruginosa* produce 3-oxo-C12 HSL, *C. sakazakii* produce 3-oxo C8, C12 HSL, C15 HSL molecules[23]. Therefore, G-citral and G-citron can be used to inhibit QS mediated through these organisms.

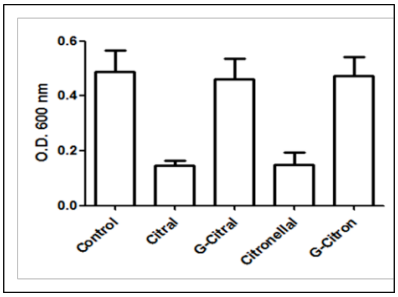


Figure 2b.2: Effect of 1 mg/ml concentration of G-citron, G-citral, citronellal, and citral on the growth of *A. tumefaciens* NTL4 (pZLR4)

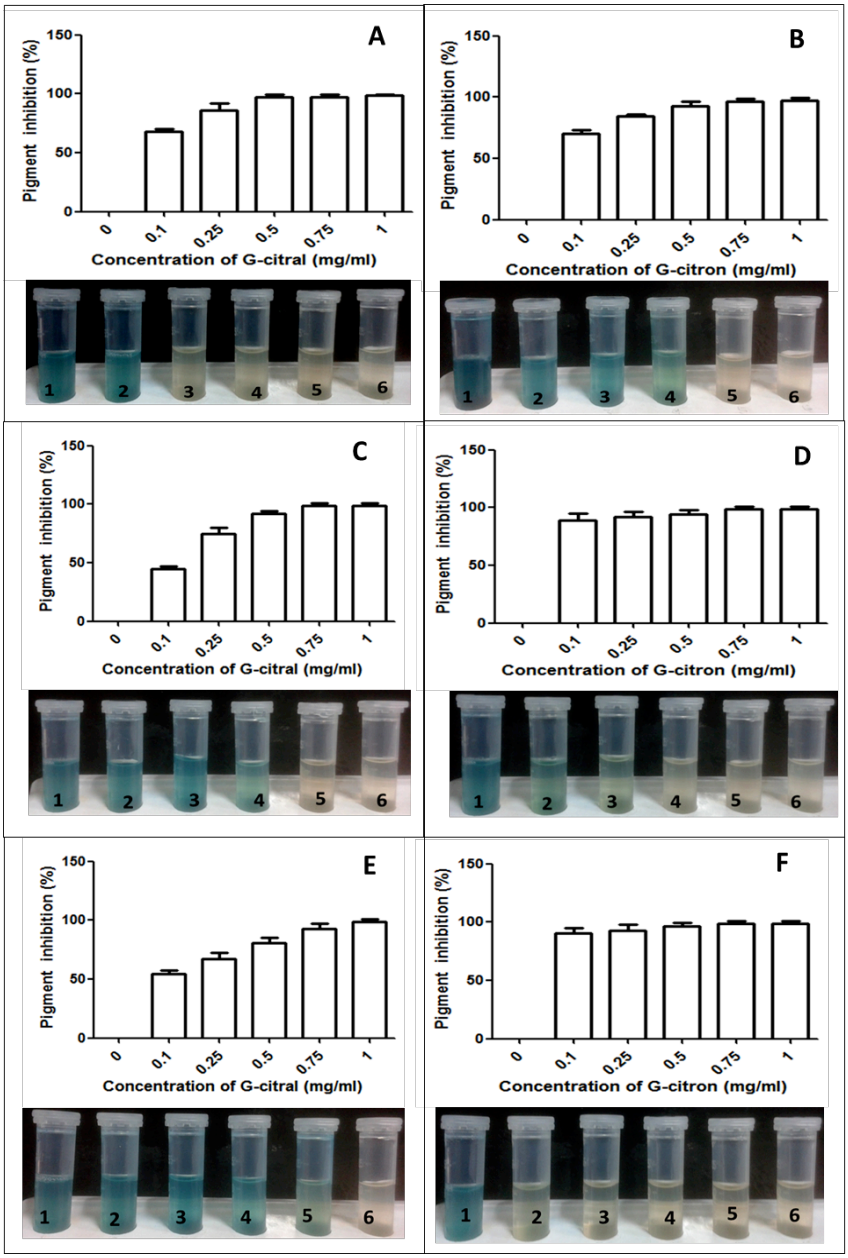


Figure 2b.3: Quantitative analysis of pigment inhibition in *A. tumefaciens* NTL4(pZLR4) by G-citral and G-citron and effect of optimum concentration on growth of *A. tumefaciens* NTL4(pZLR4): data is represented as the percentage of

pigment inhibition. Mean values of triplicate independent experiments and SE are shown. A and B: Pigment inhibition in the presence of 3-oxo-C8-HSL as external signal molecule due to G-citral and G-citron respectively; C and D: Blue pigment inhibition in the presence of C12-HSL as external signal molecule due to G-citral and G-citron respectively; E and F: Blue pigment inhibition in the presence of C10-HSL as external signal molecule due to G-citral and G-citron respectively

2b.3.3 Antibiofilm activity of glycomonoterpenes using *P. aeruginosa*, *C. sakazakii* and *V. cholerae* as test organisms:

The formation of bacterial biofilms is largely governed by quorum sensing. Countering bacteria that possess the capacity to form biofilms is of prime significance in treating chronic infections. In this context, the antibiofilm activity of glycomonoterpenes (G-citral and G-citron) was assessed by using *V. cholera* (MTCC 0139), *C. sakazakii* (ATCC12868) and *P. aeruginosa* (NCIM 5029) as test organisms. Both G-citral and G-citron showed remarkable antibiofilm activity after 24 h at 1 mg/ml concentration which was observed after staining the biofilm with crystal violet. (Figure 2b.4 B, D, F). Quantitative antibiofilm assay showed that 0.25 mg/ml G-citron and 0.5 mg/ml G-citral were sufficient to achieve more than 50 % biofilm inhibition when tested against *P. aeruginosa, C. sakazakii*. However, In the case of *V. cholerae* biofilm, G-citron showed more than 50 % biofilm inhibition at 0.5 mg/ml, and 0.75 mg/ml G-citral was required to achieve the same inhibition. We have also quantified antibiofilm activity of G-citral and G-citron with *P. aeruginosa, V. cholerae* and *C. sakazakii* as the test organisms. Results are shown in Figure 2b.4 A, C, E.

These results are in accordance with previous reports where they have demonstrated qualitatively that glycomonoterpene alcohols possess biofilm inhibition activity when tested with *P. aeruginosa* and *V. cholerae*. However, in this study they have not commented on activity of their products on biofilm produced by *C. sakazakii*[20].

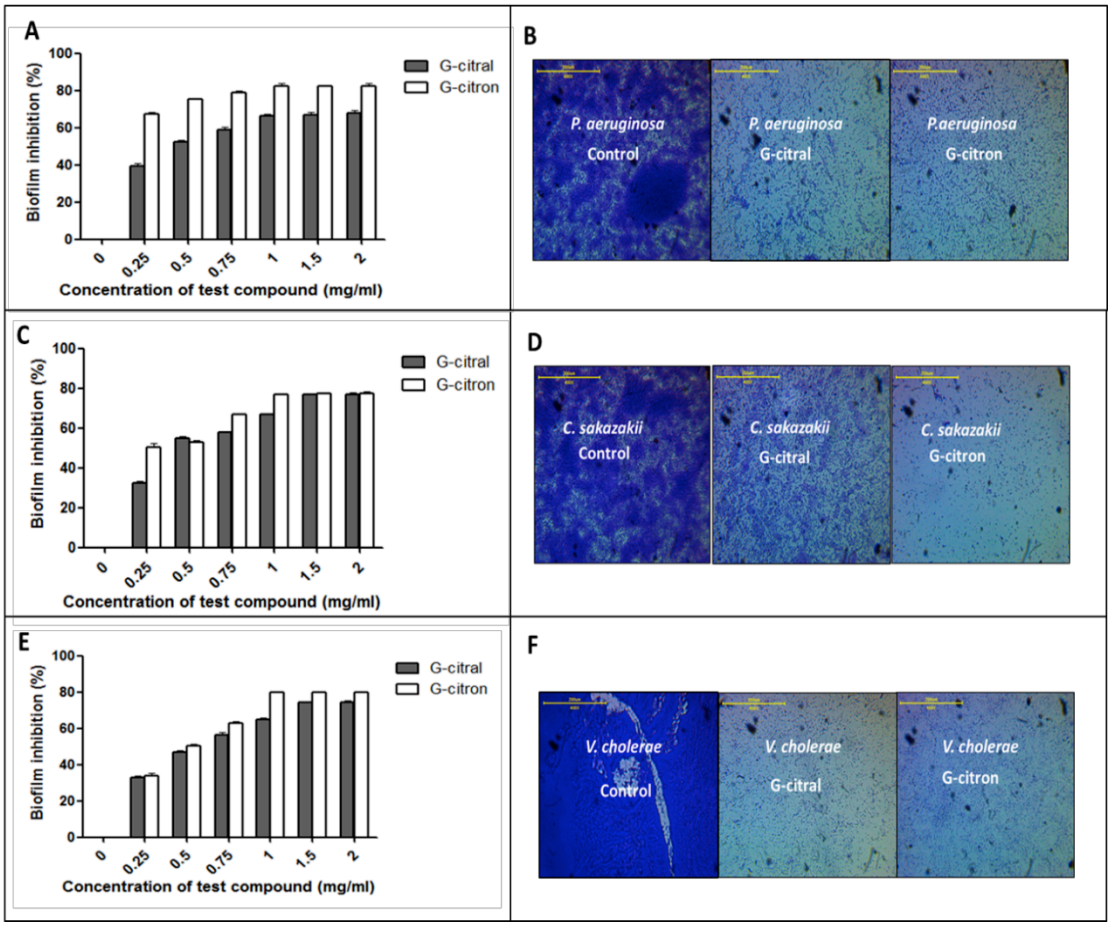


Figure 2b.4: Inhibition of biofilm formation due to G-citral and G-citron (24 h static incubation at 37 °C) Graphical representation of biofilm inhibition (on left side) and biofilms stained with 0.1 % crystal violet and observed under light microscope ×400 magnification, and scale bar is 200 μm (right side); A and B: *P. aeruginosa* biofilm inhibition; C and D: *C. sakazakii* biofilm inhibition E and F: *V. cholerae* biofilm inhibition

2b.3.4 Inhibition of Pyoverdine Production by *P. aeruginosa*:

Pyoverdine is a siderophore produced by *P. aeruginosa* (NCIM 5029) that is linked to pathogenesis. G-citral and G-citron (1 mg/ml) could achieve 65 % pyoverdine inhibition as compared to control (Figure 2b.5). This result is consistent with an earlier report where they have shown pyoverdine inhibition by glycomonoterpene alcohols in *P. aeruginosa*. Though, they have not done absolute quantification of pyoverdine they have taken O.D. of cell supernatant at 405 nm and measured pyoverdine production relative to pyocyanin production (another siderophore produced by *P. aeruginosa*)[20].

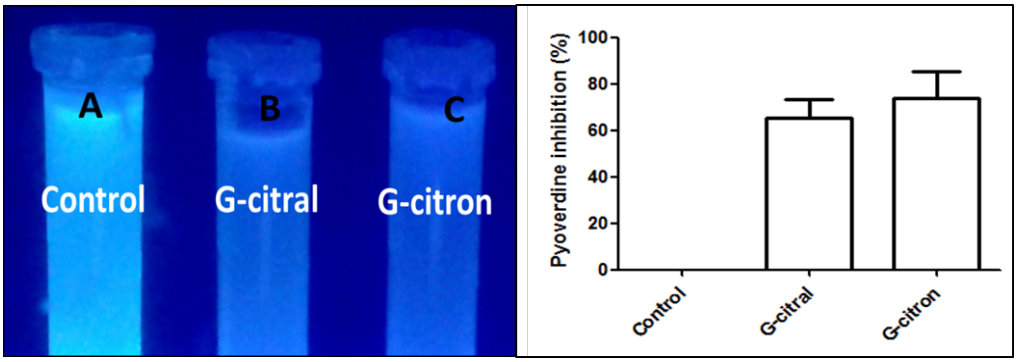


Figure 2b.5: Pyoverdine inhibition in *P. aeruginosa* by G-citral and G-citron: Decreased fluorescence in *P. aeruginosa* culture supernatant after 24 h incubation in the presence of G-citral and G-citron observed under ultraviolet light (on the left side). Fluorescence was taken at excitation wavelength 405 nm and an emission wavelength of 465 nm and represented as the percentage of pyoverdine inhibition. Mean values of triplicate independent experiments and SE are shown (right side graph)

Recently, Zhang et al. (2018) showed that essential oil components (carvone, hexanal, carvacrol, geraniol, salicylic acid, cinnamic acid, thymol, eugenol and cinnamaldehyde) inhibit biofilm formation in *Erwinia carotovora* and *Pseudomonas fluorescens* via anti-quorum sensing activity[24]. They have demonstrated *C. violaceum* CV026 assay with all above mentioned components and observed inhibition in the range of 1.27 % - 12.65 % at 0.1 mg/ml concentration but they did not mention external AHL molecules used during assay and activity of components on QS mediated through long chain AHL molecules. They have assessed biofilm inhibition activity of each compound by using *P. fluorescens* and *E. carotovora* as test organisms and could achieve maximum biofilm inhibition of 37.61% (with salicylic acid), 47.24% (with thymol) respectively. However, they did not comment on the activity of compounds on biofilm produced by *P. aeruginosa*, *V. cholerae*, *C. sakazakii* as well as pyoverdine production in *P. aeruginosa*[23].

Though, quorum sensing inhibitory activity of glyco-derivative (G-LIN, G-TER) of monoterpene alcohols (linalool, alpha terpineol) has been demonstrated earlier by using *C. violaceum* CV026 reporter strain, data was represented in the form of diameter of zone of inhibition of violacein pigment and quantification of violacein pigment was not carried out[20]. They have only qualitatively demonstrated antibiofilm activity of G-LIN, G-TER by using *P. aeruginosa* and *V. cholerae*[20].

However, in our study we have assessed QSI potential of G-citral and G-citron quantitatively and observed significant reduction in both short chain and long chain AHL mediated QS, inhibition of biofilm production by *P. aeruginosa*, *V. cholerae* and *C. sakazakii* and inhibition of pyoverdine production in *P. aeruginosa* (Figure 2b.1, 2b.3, 2b.4, 2b.5)

2b.4 Conclusion

Consequently, this chapter exclusively targeted AHL based quorum sensing system present in Gram-negative bacteria as infections caused by these bacteria impose a significant scientific challenge to get cured. Monoterpene aldehydes (the major component of essential oil) have never been transformed to hydrophilic compounds like glycomonoterpenes having unique inhibitory effects on quorum sensing, biofilm formation as well as on virulence factor production. Newly synthesized glycomonoterpenes - G-citral and G-citron, inhibited quorum sensing mediated through five AHL molecules (both short chain and long chain AHLs) and also showed significant inhibition of *P. aeruginosa*, *V. cholerae* and *C. sakazakii* biofilm and virulence factor production (pyoverdine). Overall, G-citron was observed to be more effective than G-citral. These results can be attributed to a high lactonic percentage present in G-citron (Chapter 2a Table 2a.3) Mode of action and applications of G-citron in implant and contact lens-associated infections have been shown in subsequent chapters.

Chapter 2a & 2b have been published as:

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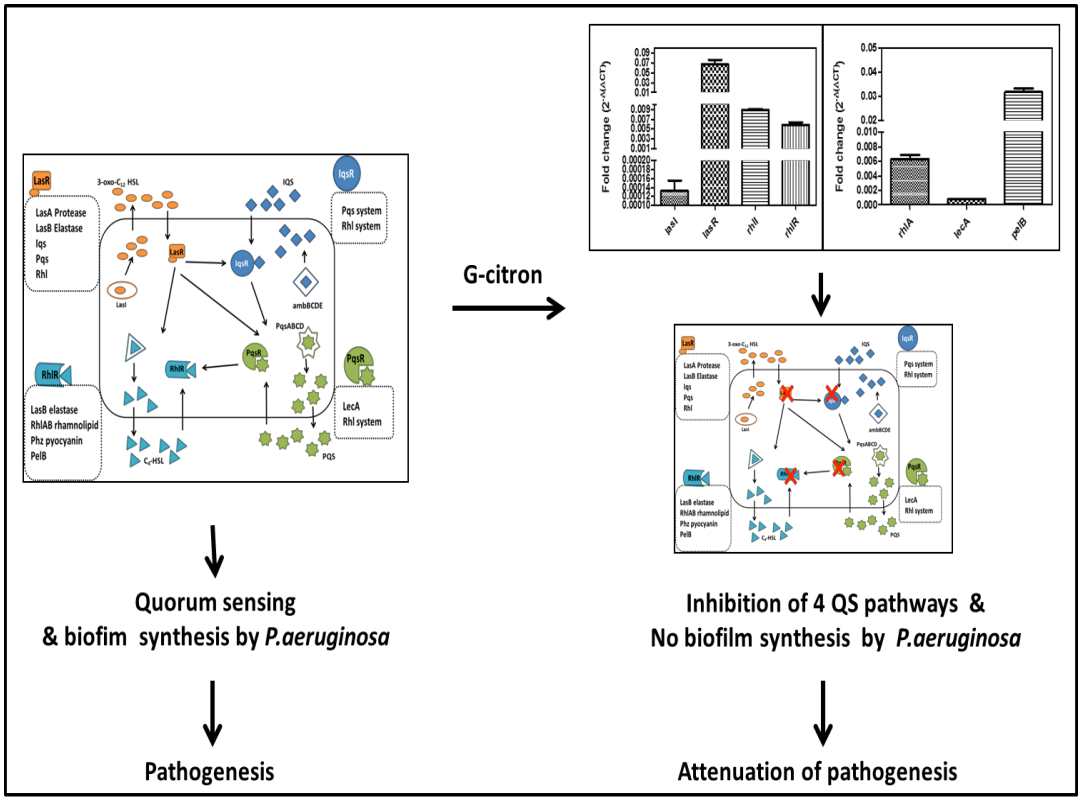
Chapter 3:

Deciphering mode of action of G-citron by studying its effect on quorum sensing related gene expression in *P. aeruginosa*.



Pseudomonas aeruginosa is a Gram-negative opportunistic and nosocomial pathogen which is majorly involved in biofilm-associated infection. According to NIH recent report, 60-80 % of infections are caused by device or non-device associated biofilm which is a consequence of quorum sensing regulated gene expression. The strategy of targeting the quorum sensing system of *P. aeruginosa* is novel approach as it does not impose selective pressure and reduces the chances of antibiotic resistance. In the previous chapter by doing bioassays we have shown that G-citron significantly inhibited quorum sensing mediated through AHL signals, biofilm formation, and virulence factor production but mechanism by which G-citron showed activity was not explored therefore in the present chapter mode of action of G-citron was found out

by using real-time PCR where G-citron (0.1mg/ml) showed >90 % inhibition in expression of QS related genes *lasI*, *lasR*, *rhlI*, *rhlR*, *rhlA*, *lecA*, *pelB*. These results offered insight into the molecular mechanism of QS inhibition by G-citron

3.1 Introduction

Gram-negative bacteria are more hazardous, and their infections are difficult to treat because of their unique cell wall features. It was also observed that C-reactive protein and IL-6 levels were significantly higher in Gram-negative bacteremia than in Gram-positive bacteremia. This suggests an early onset of immunopathophysiologic behavior of sepsis in patients with Gram-negative bacteremia[1]. *P. aeruginosa* is a Gram-negative bacterium and has received the most attention due to its higher frequency of association with numerous human diseases (Figure 3.1)[2].

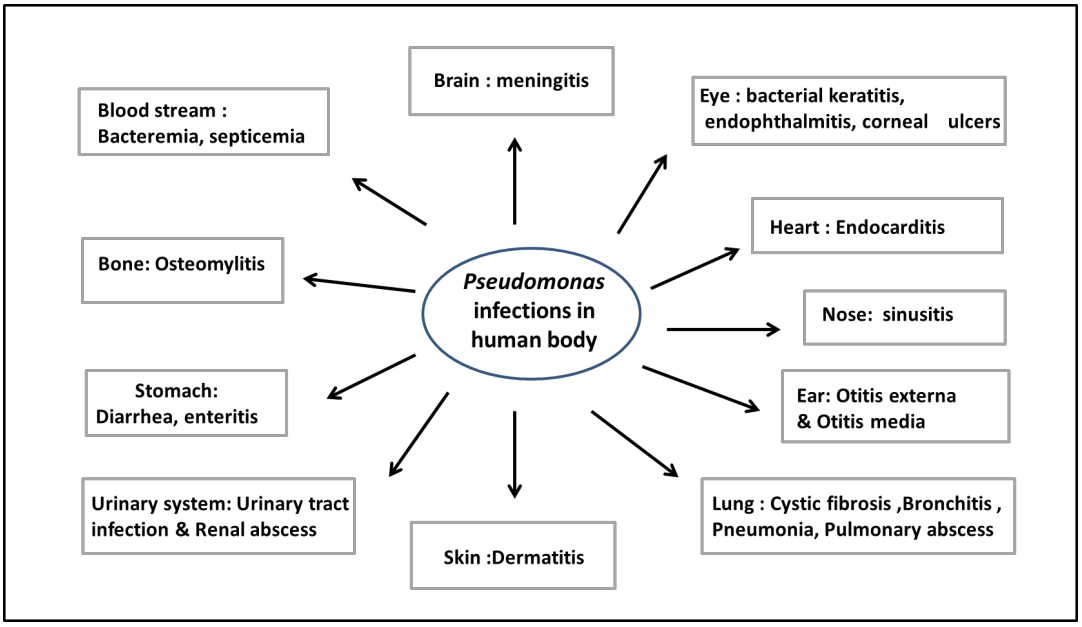


Figure 3.1: Representative examples of *P. aeruginosa* infections associated with various parts of the human body.

Patients who are immunosuppressed, particularly transplant recipients, neutropenic patients, patients with burns and with HIV, are at increased risk for *P. aeruginosa* infection[3]. *P. aeruginosa* acts as an opportunistic pathogen and is a principal etiological agent in nosocomial infections such as pneumonia, community-acquired pneumonia, urinary tract infections, bacterial keratitis-contact lens associated eye infections, chronic lung infections in patients with cystic fibrosis, infections due

to severe burns and implant associated infections[4]. The mortality rate of this infection is very high due to the amalgamation of weak host defenses, bacterial resistance to antibiotics, and the production of extracellular bacterial enzymes and toxins[2]. *P. aeruginosa* gets embedded into the self-secreted extracellular polymeric matrix called as biofilm and become resistant to antibiotics. QS systems in *P. aeruginosa* direct synthesis of biofilm, swarming motility and plays the vital role in the expression of antibiotic efflux pumps and the production of virulence factors including proteases, elastase, pyocyanin, pyoverdine, lectins, rhamnolipids, and toxins. Such virulence factors indirectly affect the development and maintenance of biofilms[5]. Thus, targeting quorum sensing is a critical step in regulating infections caused by *P. aeruginosa*.

P. aeruginosa has four quorum sensing systems, first mediated through 3-oxo-C12 HSL, second mediated through C4-HSL, third mediated through 2-heptyl-3-hydroxy-4-quinolone also known as the *Pseudomonas* quinolone signal (PQS) system and the fourth recently discovered integrated QS system (IQS) (Figure 3.2, 3.3)[5].

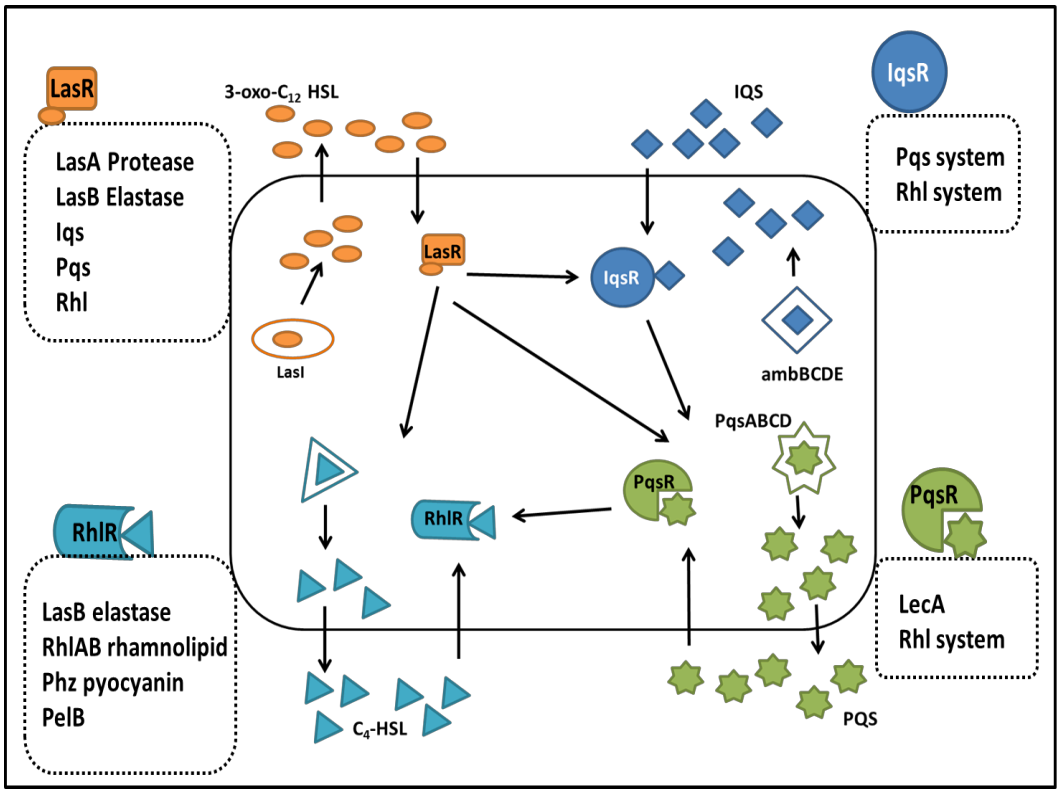


Figure 3.2 Quorum sensing pathways present in *P. aeruginosa*.

LasI/LasR and RhlI/RhlR are two major quorum sensing systems based on AHL signal molecules. LasI produces AHL signal molecule, N-(3-oxo-dodecanoyl)-L-homoserine lactone (OdDHL) which diffuses out of the cell and when it reaches critical threshold concentration it is recognized by transcriptional regulator LasR and dimer of OdDHL-LasR then binds to target promoter and induces expression of *lasI* as well as rhlR/rhlI and PQS systems (Figure 3.2) [6]. The mechanism of rhlI/R system is similar to lasI/R but consists of N-butanoyl-L-homoserine lactone (C4-HSL, BHL), AHL synthase RhlI and the cognate receptor RhlR to which BHL binds[7]. The rhlI gene, which is homologous to luxI and lasI encodes the biosynthesis of BHL and was found at the downstream of the rhlABR cluster[6]. Expression of RhlI alone could restore the production of several exoproducts such as pyocyanin, hemolysin, elastase, and rhamnolipids therefore to achieve significant inhibition of *P. aeruginosa* QS both LasI/R and RhlI/R systems should be inhibited (Figure 3.2).

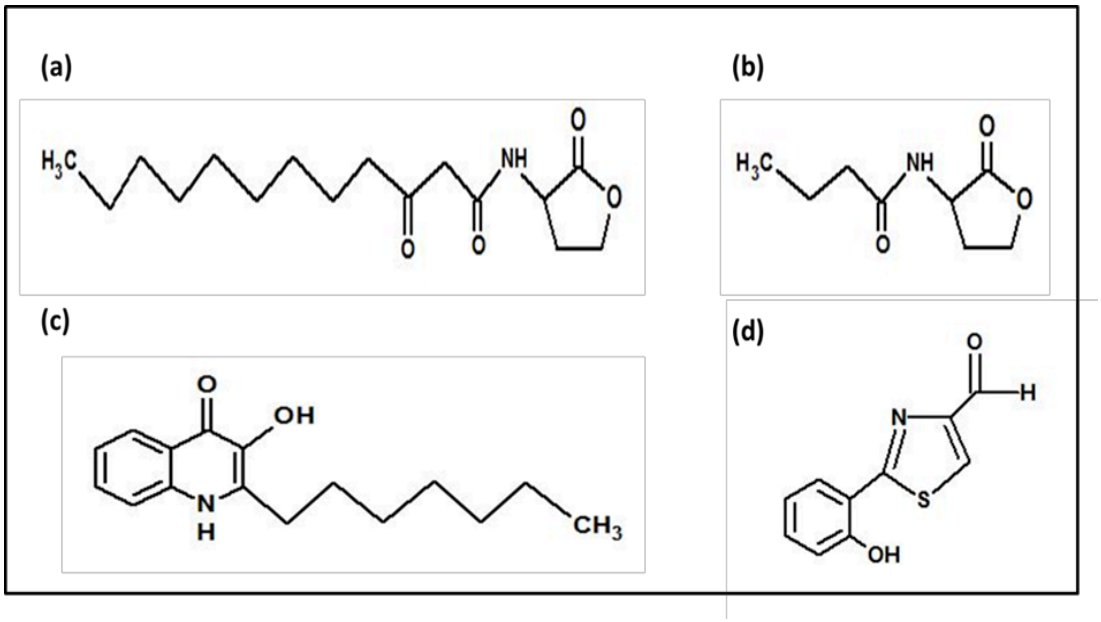


Figure 3.3 Structures of *P. aeruginosa* quorum sensing signal molecules: (a) N-(3-oxododecanoyl)-homoserine lactone (OdDHL); (b) N-butyrylhomoserine lactone (BHL); (c) 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* Quinolone Signal, PQS); (d) 2-(2- hydroxyphenyl)-thiazole-4-carbaldehyde (Integrated Quorum Sensing Signal, IQS)

The third QS system is based on PQS. PQS is chemically distinct from the AHL signals of the las and rhl systems[6]. The PQS synthesis cluster is consist of pqsABCD, phnAB, and pqsH. PqsR is a transcriptional regulator which binds to the

promoter region of pqsABCD and controls the expression of PQS. PqsR is the cognate receptor of PQS and together induces expression of PqsABCD (Figure 3.2). Fourth inter-cellular communication signal has been discovered recently named as 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS). This system integrates environmental stress cues with the quorum sensing network e.g. phosphate depletion condition. Proteins required for IQS signal synthesis is encoded by ambBCDE gene cluster. Though the signal molecule is known for IQS system, receptor is not yet discovered (Figure 3.2)[3,6].

AHL based quorum sensing system LasI/R is at the top of all four *P. aeruginosa* QS system hierarchy. The critical threshold concentration of OdDHL signal leads to the formation of dimer OdDHL/LasR. This complex further triggers expression of rhlR, rhlI, lasI. LasR-OdDHL also induces PqsR, the transcriptional regulator of the PQS biosynthesis operon pqsABCD. PQS system enhances the transcription of rhlI, thus inducing BHL production and the overall expression of the rhl QS system and also regulates rhl-dependent phenotypes. Recently, it was found that IQS system is also controlled by LasI/R under rich medium conditions. Inhibition of either lasR or lasI expression completely abrogates the expression of ambBCDE which is a synthesis cluster for the production of IQS[2].

As per recent reports, in addition to canonical BHL autoinducer, RhlR responds to an alternative ligand. This new ligand promotes a RhlR-dependent transcriptional regulation in the absence of RhlI and also found to be highly relevant in biofilms and is critical for pathogenicity in animal models of *P. aeruginosa* infection (Figure 3.4)[8]. This again indicates that inhibition of LasI/LasR system is important to inhibit RhlI independent quorum sensing network in *P. aeruginosa*.

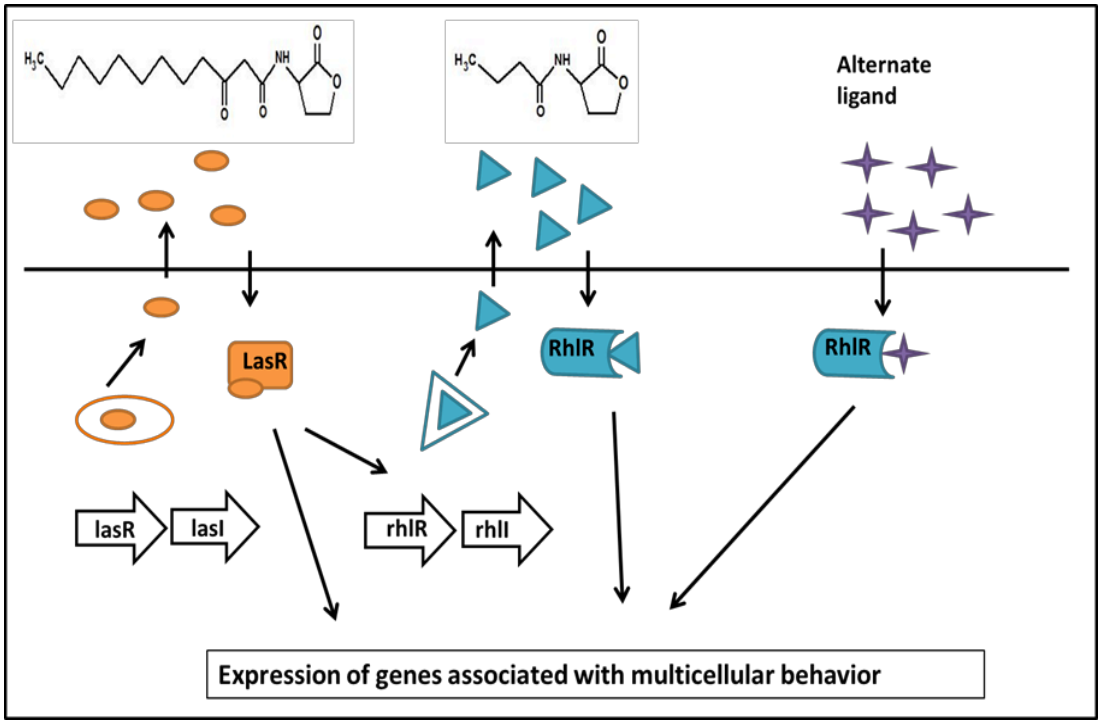


Figure 3.4 AHL and RhlI independent quorum sensing networks in *P. aeruginosa* : LasI/R (orange), RhlI/R (Blue) and one alternate ligand which binds to RhlR and enables RhlI independent RhlR function. Activation of these systems leads to expression of genes associated with group behavior.

P. aeruginosa produces virulence factors like rhamnolipid which plays a major role in biofilm maintenance, swarming motility and host invasion. These heat stable extracellular rhamnolipids can lyse polymorphonuclear leukocytes (PMNs), and monocytes derived macrophages which results in necrotic cell death. Rhamnolipids have also been found in sputum from CF patients chronically infected with *P. aeruginosa*[9]. The synthesis of rhamnolipid in *P. aeruginosa* requires two sequential reactions catalyzed by rhamnosyl transferases and first rhamnosyl transferase is encoded by the rhlAB operon. RhlA enzyme is needed to generate β-hydroxydecanoyl-β-hydroxydecanoate (HAA) which is a lipid component of rhamnolipid. RhlB coverts HAA to mono-rhamnolipid by itself. The *rhlC* gene encodes the second rhamnosyltransferase which converts mono-rhamnolipids to di-rhamnolipids. The expression of the rhlAB operon and the *rhlC* gene is regulated by the RhlI/RhlR QS system[10]. Therefore, targeting both LasR and RhlR based QS systems will significantly inhibit *P. aeruginosa* pathogenesis.

Polysaccharide encoding locus (Pel), and polysaccharide synthesis locus (Psl) play a major role in adherence, aggregation, maturation, and formation of the biofilm architecture[11]. The proteins, enzymes, and transporter molecules required for Psl and Pel synthesis are encoded by the genes *pslA-O* and *pelA-G*, respectively, in *P. aeruginosa*. Previous research showed that expression of the *pslA* gene restores the biofilm forming phenotype among mutant strains indicating its role in primary stages of biofilm formation. Psl encoded by the *pslA* gene is a neutral- charge exopolysaccharide, comprised of D-mannose, D-glucose, and L-rhamnose, arranged in pentasaccharide repeats and offers structural support during biofilm formation by both cell-to-cell and cell-to substrate attachment[12,13]. However, Pel is a glucose-rich polysaccharide polymer and is critical for maintaining cell-cell interactions in developing biofilms as well as providing protection against aminoglycoside antibiotics during biofilm growth. Thus, plays both a structural and protective role in *P. aeruginosa* biofilm[12].

In addition to above-mentioned virulence factors, *P. aeruginosa* also produces lectins LecA and LecB. LecA-mediated bacterium-cell recognition and adhesion are essential in initiating *P. aeruginosa* pathogenesis. LecA also induces a permeability defect in the host intestinal epithelium, with the subsequent increase in absorption of an important extracellular virulence factor exotoxin A. Moreover, in recent years both LecA and LecB are considered the new drug target because of new insights about their diverse roles in perturbing physiological functions of host cell[14].

So far there are very few reports on the mode of action of quorum sensing inhibitors (Table 3.1). Thus in our study to find out mode of action of G-citron in quorum sensing pathway we have used Gram-negative *P. aeruginosa* as test organism by considering its pathogenic potential. In the present work we have studied effect of G-citron on expression of 8 genes, 4 associated with QS (*lasI*, *lasR*, *rhlI*, *rhlR*) and 4 associated with biofilm formation (*rhlA*, *pslA*, *lecA*, *pelB*).

Table 3.1 Quorum sensing inhibitors and their effect on expression of genes associated with LasI/R and RhlI/R system

Sr. No.	Compound	Genes	Inhibition (%)	Concentration	Ref.
1.	6-Gingerol	*lasI, lasR, rhlR, rhlI*	74–86 %	10 µM	[15]
2.	*N*-decanoyl-L-homoserine benzyl ester (C2).	*lasR*	29.67 %,	100 µM	[7]
		lasI	21.57 %		
		rhlR	28.20 %,		
		rhlI	29.03 %		
3.	Alginate Oligosaccharide OligoG CF-5/20	*lasI*	~15 %	≥ 0.2 %	[5]
		lasR	~ 60 %		
		rhlI	~15 %		
		rhlR	~ 40 %		
4.	Delftia tsuruhatensis bacterial extract	*lasI*	~88 %	0.1 mg/ml	[16]
		lasR	~ 75 %		
		rhlI	~ 66 %		
		rhlR	~ 80 %		
5.	Aspirin	*lasI*	38 %	6 mg/ml	[17]
		lasR	72 %		
		rhlI	69 %		
		rhlR	72 %		
6.	Furanone C-30	*lasI*	~ 40 %	5 µg/ml	[18]
		lasR	Increased expression		
		rhlI	~ 10 %		
		rhlR	Increased expression		
7	*Andrographis paniculata*	*lasI*	61 %	1.25 mg/ml	[19]
		lasR	75 %		

	chloroform extract	*rhlI*	41 %		
		rhlR	44 %		
8.	Baicalin	*lasI*	~ 50 %	256 µg/ml	[20]
		lasR	~ 80 %		
		rhlI	~ 80 %		
		rhlR	~ 80 %		
9	Linolenic acid (LNA)and tobramycin (TOB)	*lasI*	~ 86 %	LNA: 0.39 mg/ml TOB: 0.078 mg/ml	[11]
		lasR	~ 75 %		
		rhlI	~80 %		
		rhlR	~80 %		
10.	Phenylalanine arginyl b-naphthylamide against U16 isolate of *P. aeruginosa*	*lasI*	69.2 %	50 µg/ml	[4]
		lasR	78.2 %		
		rhlI	93 %		
		rhlR	92.3 %		
11	5-aminolevulinic acid photodynamic therapy (ALA-PDT2	*lasI*	~85 %	20 mM	[21]
		lasR	~80 %		
		rhlI	~85 %		
		rhlR	~85 %		
12	Hordenine	*lasI*	~ 50 %	1.0 mg/ml	[22]
		lasR	~ 60 %		
		rhlI	~ 50 %		
		rhlR	~ 50 %		
13	Azitromycin(AZM)/gentamicin(GEN) /Curcumin(C) /AZM+GEN+C	*lasI*	~70/60/65/70/70 %	1/4th MIC i.e. AZM: 64 mg/ml GEN: 0.5 mg/ml C: 32 mg/ml	[23]
		lasR	~80/70/60/80 %		
		rhlI	~60/50/60/75 %		
		rhlR	~60/50/60/75 %		
14	Partially purified inhibitor factor(s)	*lasI*	50	100 µg/ml F5	[24]
		lasR	48		

	F5	*rhlI*	28		
		rhlR	29		
15	Camellia nitidissima Chi Flowers(Dichloro methane fraction)	*lasI*	~25 %	0.75 mg/ml	[25]
		lasR	~ 75 %		
		rhlI	~60 %		
		rhlR	~85 %		
16	Quercetin.	*lasI*	34 %	16 µg/ml	[26]
		lasR	68 %		
		rhlI	57 %		
		rhlR	50 %		
17	Sodium ascorbate	*lasI*	60 %	12.5 mg/ml	[27]
		lasR	80 %		
		rhlI	90 %		
		rhlR	80 %		

3.2 Materials and methods

3.2.1 Bacterial strain and growth conditions:

P. aeruginosa (NCIM 5029) was cultured in Luria Bertani (LB) broth at 37 °C for 8 h, 12 h, 16 h with and without G-citron to study its effect on expression of QS associated genes.

3.2.2 RNA isolation:

P. aeruginosa culture incubated in the presence and absence of G-citron for 8 h, 12 h, 16 h were centrifuged at 5000 rpm for 10 min at 4 °C. All sets of *P. aeruginosa* cell pellets (with and without G-citron of 3 time points-8 h, 12 h, 16 h) were washed thrice with DEPC treated water. Cell pellets were immersed in extraction buffer (1 M TrisCl pH 8.2 (1 ml), 5 M NaCl (2.8 ml), 0.5 M EDTA pH 8 (400 µl), 10 % CTAB (2 ml), DEPC water (3.8 ml), β -mercaptoethanol (30 µl)) and vortexed for 2 min and further incubated at 60 °C for 1 h. After incubation chloroform was added to each set of pellet and centrifuged at 10000 rpm for 15 min at 25 °C. To the aqueous phase collected 3 M LiCl was added, this was mixed

gently and stored overnight at -80 °C. Further, RNA isolation of each set was carried out using the RNeasy Plus mini kit (Quiagen, Hilden, Germany).

As, DNA is frequently co-extracted during the RNA isolation procedures, evaluating the presence of contaminating genomic DNA and removal of these molecules from RNA sample using DNase treatment is an essential step before real time PCR. Total RNA isolated were treated separately with DNase kit (Promega, USA) to remove traces of contaminant genomic DNA. The reaction mixture (10 µl) containing 1 µl of 10x DNase buffer, 1 µg RNA and 1 µl of DNase (1 U/µl) were incubated at 37 °C for 30 min. The reaction was stopped by adding 1 µl of DNase stop solution, and the DNase was inactivated by heating the reaction mixture at 65 °C for 10 min.

3.2.3 Quantification of RNA samples:

RNA quality as 260 nm/ 280 nm ratio was evaluated using Nanodrop 1000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

3.2.4 cDNA Synthesis:

Six microgram of DNase treated total RNA was used to carry out reverse transcription for the synthesis of cDNA using High Capacity cDNA reverse transcription kit (Applied Biosystem, Carlsbad, CA, USA). PCR reaction mixture contained 10X buffer (6 µl), 100 mM dNTPs (2.4 µl), Random primer (6 µl), Reverse transcriptase (3 µl), RNase inhibitor (3 µl) and RNA (6 µg) in MilliQ. cDNA synthesis conditions used were as follows:

Table 3.2 : cDNA synthesis conditions of PCR

Program	Step1	Step2	Step3	Step4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

Confirmation of cDNA was done using 16S rRNA as an endogenous control. Reaction mixture used was 10X Buffer (1 µl), 3 U enzyme (0.3 µl), 100 mM dNTPs (2.5 µl), 10 µM forward primer (0.5 µl), 10 µM reverse primer (0.5 µl), template (0.5 µl) and MilliQ (4.7 µl). Amplification conditions used were as follows: denaturation at 94 °C for 3 min, followed by 30 cycles of, 94 °C for 30 sec, annealing at 62 °C for

30 sec then 72 °C for 30 sec, with subsequent elongation step at 72 °C for 2 min. Amplified product (~100 bp) was checked on 2 % agarose gel.

3.2.5 Designing of primers:

Primers for quantitative reverse transcriptase PCR analysis of quorum sensing related genes (Table 3.3) were designed using NCBI primer blast tool.

Table 3.3: PCR primers for real time PCR (RT-PCR)

Gene	Primer direction	Sequence (5'-3')	Amplicon size in (bp)
lasI	Forward	CGCACTCAGTCCTTATTACA	112
	Reverse	GAAGGTGTTCTTCAGCATGT	
lasR	Forward	CTCAAGTGGAAAATTGGAGTG	118
	Reverse	GAAGGCGTTCTCGTAGTC	
rhlI	Forward	AAGGACGTCTTCGCCTAC	118
	Reverse	AGAATATCTTCATCGCCAGC	
rhlR	Forward	CTTGGTCATGATCGAATTGC	99
	Reverse	TTCTCGATGAAGACCTGATG	
rhlA	Forward	GAGACCGTCGGCAAATAC	107
	Reverse	TGGTCGATGTGAAAGCG	
pslA	Forward	ATACGACCTCGAATACATCG	100
	Reverse	TCAGTAGACTTCCTTGGTCA	
lecA	Forward	GTTCTGGCTAATAACGAAGC	107
	Reverse	TTCTGGGTAGGTCCGTAAC	
pelB	Forward	GTTCTACGAGGTGGAACTG	118
	Reverse	GTTCAACGAAACGTGCTC	
16S rRNA	Forward	GAATCTGCCTGGTAGTGGGG	110
	Reverse	ATCCCCGACTTTCTCCCTCA	

3.2.6 Quantitative reverse-transcriptase PCR:

Quantitative reverse-transcriptase PCR was performed on instrument Applied Biosystems (California, USA) using the Fast Start Universal SYBR Green master mix (Roche Inc. Indianapolis, Indiana, USA) and *16S rRNA* gene as an endogenous reference gene[20] employing the primers mentioned in Table 3.3. Four

QS associated genes (*lasI, lasR, rhlI, rhlR*) and four genes related to QS inducible phenotypes (*rhlA, pslA, lecA, pelB*) were selected, and the expressions of these genes were quantified in *P. aeruginosa* cells treated with 0 (control) or 0.1 mg/ml (Test) G-citron. Each reaction mixture consisted of the following reagents: 5 µl SYBR Green, 0.5 µl each of the forward and reverse primers (5 µM), 4 µl template RNA (1:20 dilution), and RNase free water to generate a 10 µl final volume. The thermal profile of the RT-qPCR for each of the target genes was as follows: initial denaturation at 95 °C for 10 sec (stage 1), followed by 40 cycles of denaturation at 95 °C for 3 sec, annealing at 60 °C for 30 sec (stage 2). Relative quantification (ΔΔCT method) was carried out manually by using the following formulae [28].

$\Delta Ct_{control}$ = Ct (gene of interest in control) – Ct (16s rRNA gene in control)
ΔCt_{test} = Ct (gene of interest in test) – Ct (16s rRNA gene in test)
$\Delta\Delta Ct = \Delta Ct_{test} - \Delta Ct_{control}$
and

Fold gene expression $= 2^{-(\Delta\Delta Ct)}$

where, Expression of gene of interest in test $= 2^{-(\Delta\Delta Ct)} \times$ Expression of gene of interest in control

Statistical analysis was done using Graph pad prism 5 software[29]. Six sets of samples as described above from three independent biological replicates were used for this analysis.*16S rRNA* gene was chosen as an internal control to normalize the real-time RT-PCR data and to calculate the relative fold changes in gene expression. Expression of the individual gene in different time points of control was considered as 1 and, relative gene expression in G-citron treated cells were reported. Experiments were repeated independently three times with different RNA samples each time.

3.3 Results and discussion:

3.3.1 RNA isolation and cDNA synthesis:

Total RNA was isolated from each set of control and test incubated for 8 h, 12 h, and 16 h. DNase treatment successfully removed genomic DNA contamination from isolated total RNA (Figure 3.5 (a)). The concentration of total RNAs from each set was found to be in the range of 1-2 µg/µl with 260 nm/ 280 nm ratio ~2 which indicated the purity of isolated total RNA. cDNA samples amplified with 16s rRNA primer showed amplification product of ~100 bp (Figure3.5 (b))

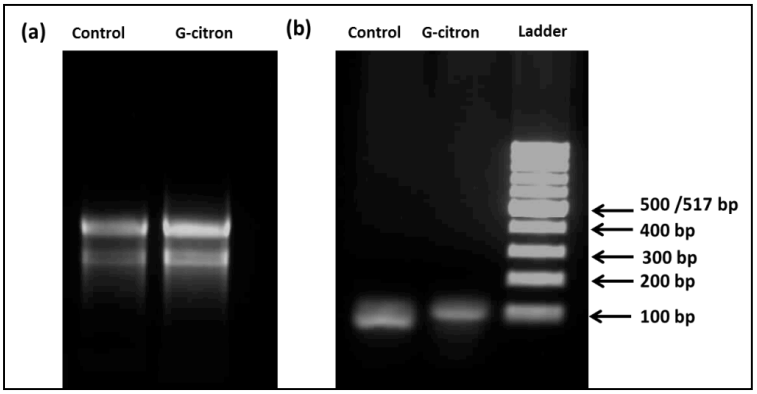


Figure: 3.5 Agarose gel images of RNA and cDNA samples: **(a)** RNA samples isolated from *P. aeruginosa* incubated with and without G-citron (0.1 mg/ml) were separated by electrophoresis in 1 % non-denaturing agarose gel and stained with bromophenol blue **(b)** cDNA samples of *P. aeruginosa* incubated with and without G-citron (0.1 mg/ml) synthesized with random primers and further amplified using 16s rRNA.

3.3.2 Analysis of quantitative reverse-transcriptase PCR:

Transcript levels of the selected genes in the control and test sets at 8 h, 12h incubation were significantly low. However, control sets at 16 h incubation showed significantly detectable expression of selected genes except *pslA* gene. This depicts that initiation of quorum sensing pathway in *P. aeruginosa* (NCIM5029) occurred after 12 h of incubation and expression of *pslA* gene must be initiating after 16 h. The expression of the *16S rRNA* housekeeping gene was not affected by G-citron. At a concentration of 0.1 mg/ml, G-citron inhibited the expression of QS associated genes *lasI* (99.9 %), *lasR* (93.3 %), *rhlI* (99.1 %) and *rhlR* (99.4 %) resulting in the termination of the LasI/R and RhlI/R system (Figure 3.6). In addition to this at the same concentration (0.1 mg/ml) G-citron also inhibited expression of genes which are mainly involved in QS associated phenotypes, e.g. biofilm. *rhlA* (99.4 %), *lecA* (99.9 %), *pelB* (96.8 %) (Figure 3.7). Expression inhibition of quorum sensing gene in G-citron treated *P. aeruginosa* (%) was calculated with the following formula:

Expression inhibition (%) = $(1- 2^{\Delta(\Delta CT)}) \times 100$

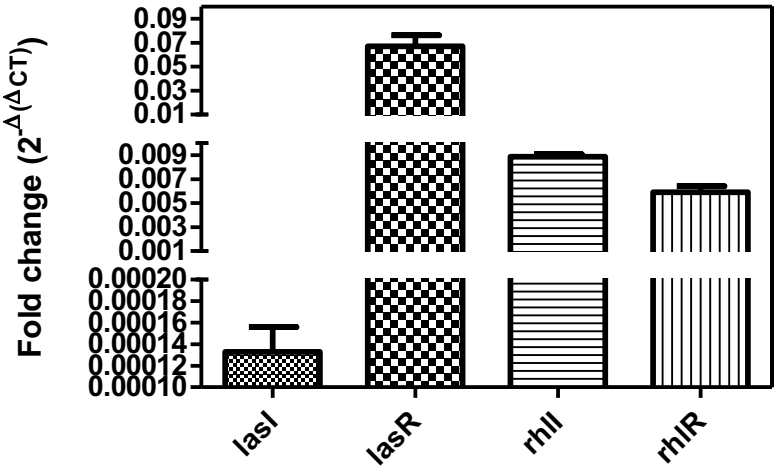


Figure 3.6 Graph elucidating fold change in expression of *lasI*, *lasR*, *rhlI* and *rhlR* gene

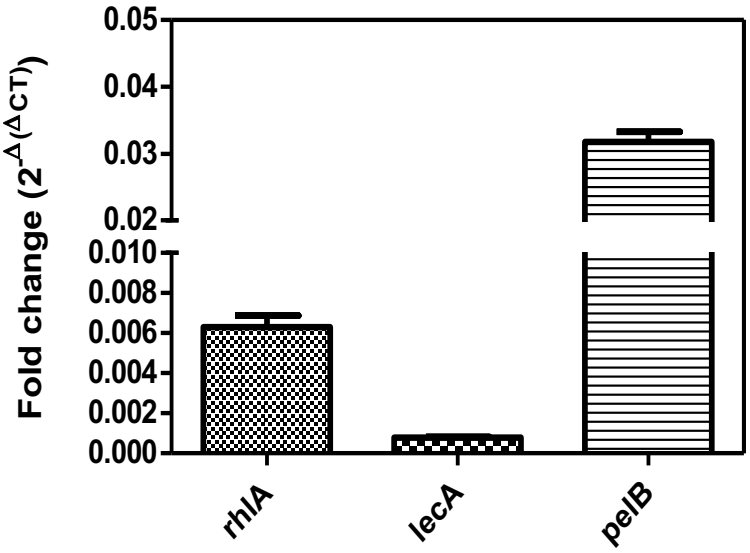


Figure 3.7 Graph illustrating fold change expression of *rhlA*, *lecA* and *pelB* gene.

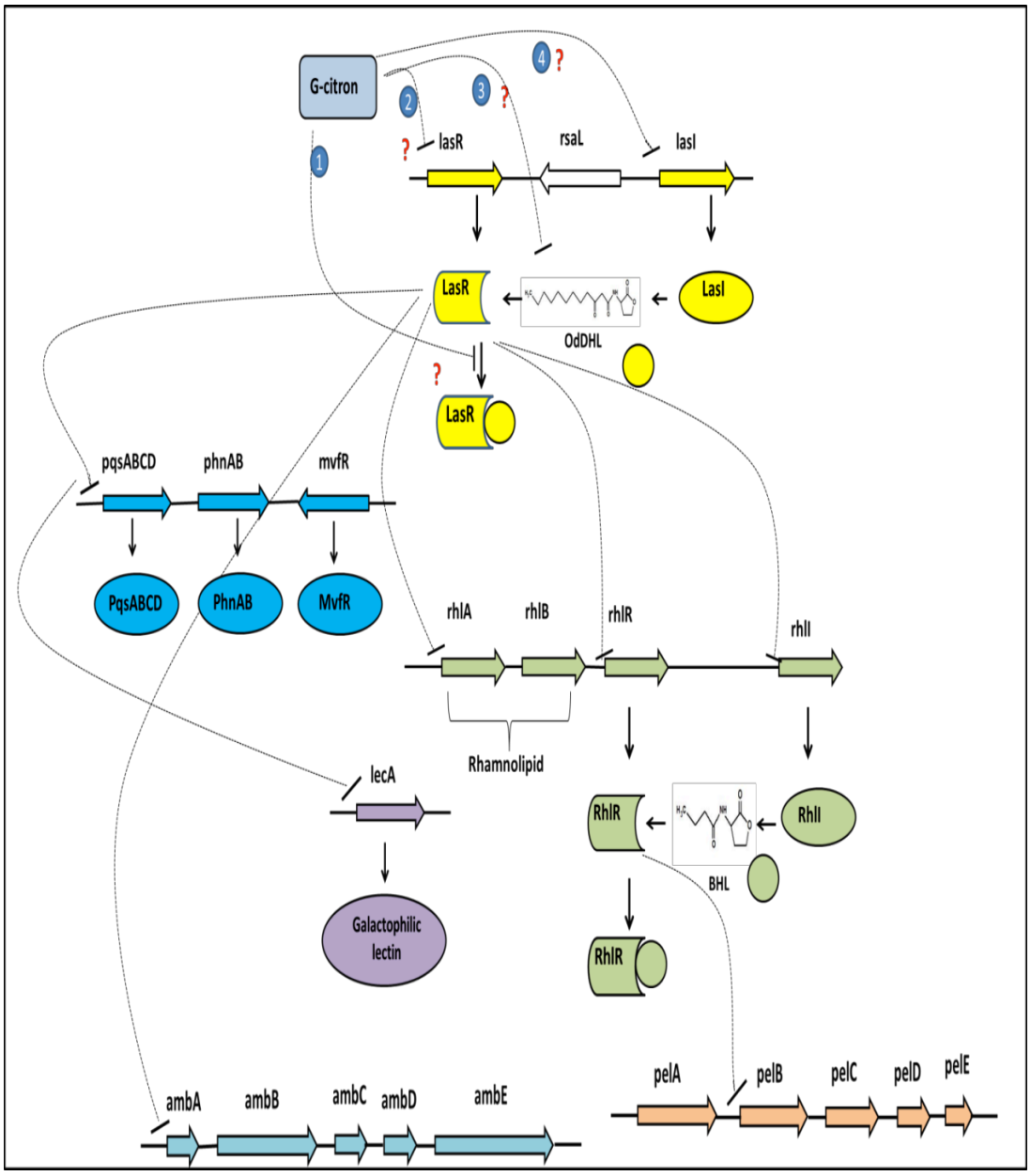


Figure 3.8 A schematic depicting alteration of QS system and virulence factor production caused by G-citron within the LasI-LasR, RhlI-RhlR, PQS and IQS quorum sensing systems and those related to the production of virulence factors (e.g., rhamnolipid, pellicle and lectin synthesis). Negative regulations of the gene expression are indicated as dotted lines.

As G-citron inhibited LasI/LasR QS system which is considered at the top of all four *P. aeruginosa* QS system hierarchy it must have inhibited other non AHL based quorum sensing system to some extent. This assumption got confirmed by our other result where we observed inhibition of *lecA* gene expression at 16 h due to G-citron (Figure 3.7). Expression of *lecA* gene is usually activated by activation of PQS

quorum sensing system and as per our results; G-citron has inhibited expression of *lecA* gene with 99.9 % (Figure 3.7). Similarly, IQS system normally activates PQS and Rhl QS systems (Figure 3.2)[6]. But in our study, G-citron inhibited expression of *rhlA* (99.4 %) and *lecA* (99.9 %) (Figure 3.7) genes whose expression is induced by activated RhI/R and PQS quorum sensing systems respectively (Figure 3.2). This indicates that G-citron also inhibited non AHL quorum sensing system (PQS and IQS).

Therefore, it can be concluded that, G-citron must be acting on quorum sensing pathway of *P. aeruginosa* with four different possibilities which subsequently inhibit other QS systems. First, it may inhibit binding or dimerization of 3-oxo-C12 HSL to LasR receptor, second it may inhibit synthesis of LasR, third G-citron may modify signal molecule, or fourth it may inhibit synthesis of LasI (as shown in schematic Figure 3.8). Consequently, inhibition of LasI/LasR may lead to inhibition of all other QS systems (Figure 3.8). From our results, probability of G-citron's activity on individual QS system can't be ignored. So this activity of G-citron can be checked further by using *P. aeruginosa* mutant strains of LasI/R or both LasI/R and rhlI/R QS systems.

Complete understanding of QS associated gene targets of quorum sensing inhibitors is crucial for its application. Though many quorum sensing inhibitors have been reported so far very few have been studied in detail for its effect on QS associated gene expression. As LasI/R and RhlI/ RhlR are two major QS pathways present in *P. aeruginosa*, some representative reports in which effect of QSI on these two pathways is mentioned are summarized in Table 3.1. As per results reported so far, only phenylalanine arginyl b-naphthylamide (50 μg/ml) and sodium ascorbate (12.5 mg/ml) have shown ≥ 90% expression inhibition of RhlI/ RhlR QS system (Table 3.1)[4,27]. However, these compounds did not inhibit LasI/LasR QS system with similar expression inhibition (%). Conversely, G-citron (100 μg/ml) inhibited both the AHL QS system of *P. aeruginosa* with > 90% expression inhibition of *lasI* (99.9 %), *lasR* (93.3 %), *rhlI* (99.1 %) and *rhlR* (99.4 %). Hence, G-citron can be considered among best quorum sensing inhibitors reported so far.

3.4 Conclusion

As, *P. aeruginosa* pathogenesis is mainly attributed to its four interconnected signaling mechanism, QS inhibition is a promising tool for the treatment of *P. aeruginosa* infections. Our study clearly demonstrated that G-citron inhibited expression of 4 genes associated with QS (*lasI*, *lasR*, *rhlI*, *rhlR*) and 3 genes linked with QS mediated phenotypes (*rhlA*, *lecA*, *pelB*). The current study suggests that G-citron has the potential to be used as individually or in combination with antibiotics to treat *P. aeruginosa* infections. However, further studies regarding the application of this compound are required, in order to confirm the cytotoxicity, stability, and activity of the compound *in vivo*. This study showed for the first time mode of action of G-citron (glycomonoterpene) synthesized from citronellal (monoterpene aldehyde).

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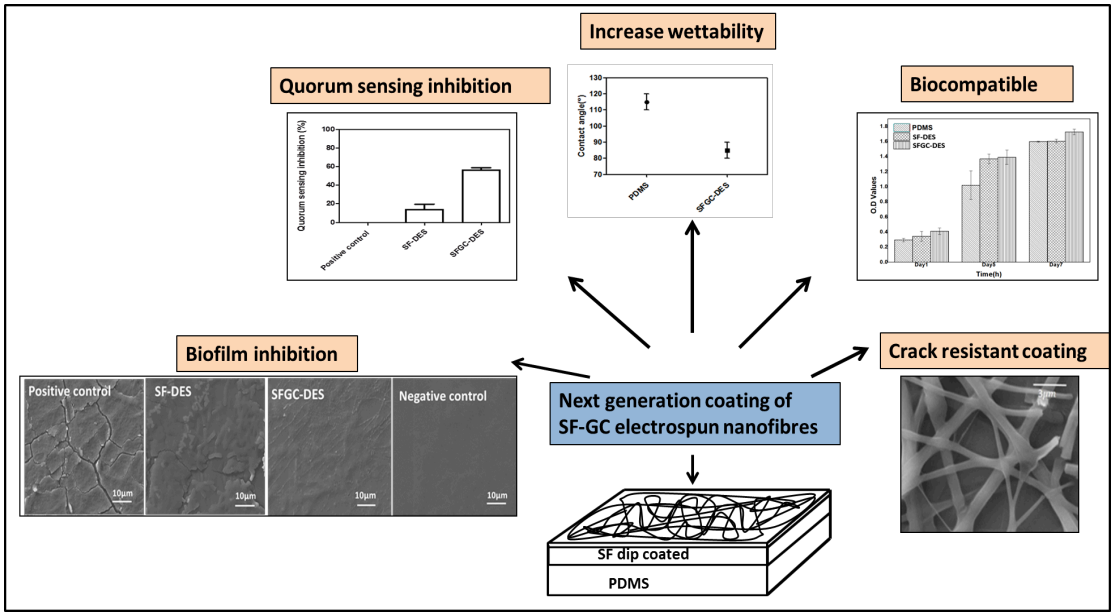
Chapter 4a.

Development of novel G-citron based surface coating for biomedical implants



The development of novel surface coatings for biomedical implants with reduced chances of premature failure is the most pressing concern. Polydimethylsiloxane (PDMS) is a widely used implant material due to its attractive device fabrication properties in the biomedical field. However, in many cases, it suffers premature failure which usually initiates due to three interlinked reasons first, bacterial biofilm formation second non-specific protein adsorption leading to foreign body response and third mechanical deformation such as delamination, cracking, crazing, etc. either during surgical handling or during use. In this chapter, we have used a green biomolecule G-citron whose synthesis and properties have been mentioned in the previous chapters and can be considered as the next generation antibiofilm molecules on account of its quorum sensing inhibitory activity. G-citron was blended with silk fibroin (a natural biocompatible protein polymer) by using a unique process which resulted in the formation of a coating that inhibits biofilm formation, inhibits adsorption of protein due to the hydrophilic surface and is stable under mechanical stresses in tensile, torsion and bending modes. The coating process

involves a combination of two conventional coating processes – namely dip coating and electro-spinning. We have demonstrated that the quorum quenching activity of the biomolecule is retained during the processing steps and the coating exhibited excellent antibiofilm activity against common infection-causing bacteria *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*.

This coating of G-Citron -SF electrospun nanofibres provides a novel and nonbiocidal approach to the design of coatings that attenuate bacterial virulence as well as foreign body response. We anticipate that this approach has the potential to be used as a coating material on various polymers similar to PDMS and will act as next-generation coating for the long-term success of implantable medical devices.

4a.1 Introduction

Every year millions of people improve their quality of life by using diagnosis, treatments, surgeries which involve implantable medical devices. However, increasing failure rate of these implantable medical devices is a prime concern[1]. The failure of these implants has primarily been attributed to three interlinked factors - device-associated bacterial infection, foreign body response and mechanical deformation of implants[2]. More than 25 % of the hospital-acquired infections (HAI) are directly linked to the implanted devices[3], for example, urinary tract infections (UTIs) are thought to constitute 30−40 % of HAIs worldwide, and more than 80 % of them are directly associated to catheterization, viz. catheter-associated UTIs (CA-UTIs)[3]. Similarly, 87 % of bloodstream infections are directly caused by intravascular devices, including pacemakers, implantable cardioverter defibrillators, left ventricular assist devices and prosthetic vascular grafts; and 86 % of pneumonia conditions are due to mechanical ventilation[1]. Bacterial biofilms and antibiotic resistance have found to be dominant problems in treating such infections[4]. As per the Centre for Disease Control and Prevention (CDC) and National Institute of Health (NIH), the 65-80 % of infections are caused by bacterial biofilms[1]. Bacteria get embedded into self-secreted extracellular polysaccharide (EPS) matrix called as biofilm and protect themselves from antibiotics. This has fuelled to the global problem of antibiotic resistance. This biofilm formation phenomenon is usually directed by their ability to communicate with other bacteria called as quorum sensing (QS). Inside the EPS matrix, QS play an important role in the biofilm organization as well as in the production of virulence factors that facilitate attachment to the host cells. In the case of Gram-negative bacteria, QS is mainly achieved through N-acyl homoserine lactone (AHLs) However,

Gram positive bacteria use small peptides as QS signal molecules[5]. Implant infecting *P. aeruginosa* and *S.epidermidis* have high rates of antibiotic resistance due to their biofilm forming capacity. More than 40 % of *S. epidermidis* strains isolated from postsurgical implant-related infections were found to be resistant to gentamicin[4]. Therefore, biofilm formed due to quorum sensing is a critical factor in implant failure[5].

The second most common reason for the failure of implants is the foreign body reaction and formation of a fibrotic capsule. Capsular contracture or implant failure due to fibrotic capsule formation has been reported during the first year of the breast reconstruction and augmentation surgeries with the rate of 4.3–26.9 % [6]. The implant surface is hydrophobic. This leads to the anchoring of various proteins onto the implant surface[2]. This event causes a catastrophic chain reaction that first leads to activation of neutrophils which further attracts macrophages and monocyte precursor cells to the implant surface. These macrophages secrete few factors which in turn, signal fibroblasts in the patient's body to adhere and produce collagenous fibrotic capsule around the implant. Subsequently, it impedes metabolite transport, healing, device-tissue integration and creates surface susceptible to bacterial infection[7]. So the crucial aspect here is the hydrophobicity of the implant surface that leads to protein adsorption.

The third factor causing implant failure is the mechanical deformation of implants. Problems related to flaking, delamination, cracking and crazing of coatings are key challenges in front of successful implantation of medical devices. Recently, FDA recalled polytetrafluoroethylene coated guide wires as separation of the coating might cause severe injuries to the patient both due to the migration of coating to other body parts and exposure of underlying medical device surface which could trigger other undesirable events such as blood clots[8].Researchers have also reported microcracks in coatings especially when subjected to mechanical deformation. The failure of coatings may be attributed to the mechanical stresses imposed on the implants due to handling either during manufacture, packaging and also during surgical intervention by the medical practitioners. Some coatings may also fail after implantation as catheter tubes, for example, experience a variety of bending/tensile stresses when in use[9]. As the inherent bulk properties of the implant remain unaltered. Surface coatings are, therefore, a rapidly evolving field.

Thus, the key for the successful implantation could be a use of the active compound in coatings which possess antibiofilm activity with reduced chances of antibiotic resistance and surface modification to avoid foreign body response, mechanical deformation of implants by using unique coatings of biopolymers.

Poly-dimethyl siloxane (PDMS) is the most widely used synthetic polymer for biomedical applications today due to its unique properties such as ease of fabrication, optical transparency, elastomeric properties, and gas permeability low manufacturing cost, biocompatibility, and low toxicity. However hydrophobic nature of PDMS surface cause low wettability of surface and makes the PDMS devices more susceptible to bacterial as well as non-specific protein adhesion[10,11]. Therefore, medical grade Polydimethylsiloxane (PDMS) is chosen as the substrate for our study to improve surface properties.

As mentioned earlier, bacteria forming biofilm protect themselves and become resistant to the higher dosage of antibiotics and in addition to this, as antibiotics put selective pressure by killing bacterial cells use of antibiotics to treat implant-associated infections is increasing the burden of antibiotic resistance[12]. However, unlike antibiotics quorum sensing inhibiting molecules (QSI) do not put selective pressure by killing bacterial cells instead inhibits biofilm formation and pathogenesis by interrupting QS i.e., cell to cell communication. Therefore, bacteria are less likely to develop resistance to quorum sensing inhibitors in the future[12]. Along with this, upon administration of QSI the cells remain in planktonic form, and they are available to be killed by phagocytic cells in the body, without affecting the normal flora. This also avoids infections by opportunistic pathogens and justifies QSIs as next-generation molecules[13]. Thus, in present work, we demonstrate the incorporation of a QSI biomolecule – G-citron (GC) to inhibit the bacterial biofilm formation on implants. As mentioned in an earlier chapter, GC is biosynthesized from citronellal and glucose by a non-pathogenic yeast *Candida bombicola*. Citronellal was mainly chosen as a substrate for bio-modification because it is a major component of many readily available essential oils. The modification with glucose results in a molecule with improved hydrophilicity and hence enhances its bioavailability.

In 2014, Thomas Schiebel and co-workers showed that the spider silk coating inhibited foreign body response by hindering fibroblast proliferation, collagen I synthesis, and differentiation of monocytes into CD68-positive histiocytes[6] and In the last few years natural silk fibroin (SF) has emerged as a promising biomaterial on

account of easy availability, proven biocompatibility, biodegradability, and aqueous processability[14–16]. Therefore, along with GC, natural silk fibroin (SF) obtained from *Bombyx mori* silkworm is chosen in our study as the biomaterial for coating application considering that SF will attenuate biological events leading to fibrosis, thereby acting as a bio-protectant in implants. Various techniques have been used to deposit SF coatings on both polymer as well as metal scaffolds[17–19]. However, the mechanical properties of the coating and its ability to resist mechanical deformation have not been quantified and studied.

Thus, it is an objective of this work to provide an innovative solution to simultaneously resolve multiple causes for failures of implant devices – biofilm formation, foreign body response and cracking/delamination of the coating.

4a.2 Materials and Methods:

4a.2.1 Preparation of PDMS discs

Medical grade PDMS (Sylgard 184, Dow Corning) was cast on polystyrene petri plates to obtain a disc with a uniform thickness of 1mm. The prepolymer was thoroughly mixed with the curing agent for 5 min using a weight ratio of 10:1. It was then poured into the petri plate and degassed for 30 min. This mixture was kept in a convection oven at 40 °C for 24 h. Circular PDMS discs of required diameters were then cut out and used for further experimentation. A cleaning process of 30 min sonication in isopropyl alcohol (IPA) was followed to remove dust particles before further experimentation. This process of cleaning was followed by a drying step under vacuum at 60 °C for 4 h to remove any traces of the IPA solvent.

4a.2.2 Oxygen plasma treatment of PDMS disc

The plasma treatment was done on both sides of the PDMS disc using Emitech 1050 plasma unit. The chambers were initially purged for 15 min with oxygen gas. The optimized plasma conditions of 50 W RF power and 1 min time duration were determined by measuring the contact angle on the surface and confirming no physical damage to the PDMS disc surface using optical microscopy. The plasma-treated discs were stored under DI water before use for further experimentation.

4a.2.3 Synthesis of glycomonoterpene

Glycomonoterpene (GC) was synthesized as per method given in Chapter 2a.2.3. Briefly, *C. bombicola* ATCC 22214, non-pathogenic yeast cells were grown in MGYP broth for 48 h at 28 °C. The yeast cells were then transferred to a production medium containing 10 % Glucose and citronellal (monoterpene). The cells were incubated in this medium at 28 °C for 7 days and the product formed at the end of 7 days was harvested by ethyl acetate solvent extraction.

4a.2.4 Preparation of SF solution

The silk fibroin solution was prepared from *B. mori* silkworm cocoons obtained from Central Sericultural Research and Training Institute, Mysore as per the protocol described elsewhere[20]. Briefly, the cocoons were degummed using 0.5 wt% sodium bicarbonate ($NaHCO_3$) solution to remove the sericin protein. The dissolution of the cottony mass of fibroin fibers was done using a chaotropic salt Lithium Bromide (LiBr) at 60 °C for 4 h. The salt was then extensively dialysed out to obtain regenerated silk fibroin (RSF) solution with approximately 3-5 w/v% concentration. The RSF solution was then lyophilized at -55 °C to obtain SF powder. This powder was stored at -20 °C until further use. The powder was dissolved in Hexafluoroisopropanol (HFIP) at room temperature to obtain a 5 wt/v% solution and this solution was then used for electrospinning[21].

4a.2.5 Preparation of GC and silk fibroin coating

The plasma-treated PDMS disc was used for coating experiments with GC-SF. The RSF solution was diluted to 0.4 w/v % using DI water. The disc was immersed in this solution for 10 min to obtain a uniform SF coating. Further, the PDMS disc was taken out of the SF solution and immediately mounted onto the collector plate of an electrospinning apparatus before drying. The electrospinning was done using 5 w/v% SF/HFIP solution as shown in Figure 4a.1. The tip to collector plate distance was maintained at 10.5 cm, and a voltage of 30 KV with a flow rate of 1 ml/h was used for electrospinning. After electrospinning, the discs were kept for drying at 40 °C for 12 h to remove traces of the solvent. This was followed with a methanol vapor annealing treatment to introduce stable beta-sheet conformation in the protein. The coated discs were incubated in a saturated methanol vapor closed chamber for 48 h at room temperature. Further characterization was done after this treatment. These discs are

further referred to as SF-DES (Dip + Electrospinning)[22]. Similarly, blends of SF and GC were made in 1:3 ratios to form 5 wt/v% solution in HFIP. This solution was then electrospun on the SF dip-coated PDMS disc and the coated discs were incubated in a saturated methanol vapor closed chamber for 48 h at room temperature. These discs are further referred to as SFGC-DES.

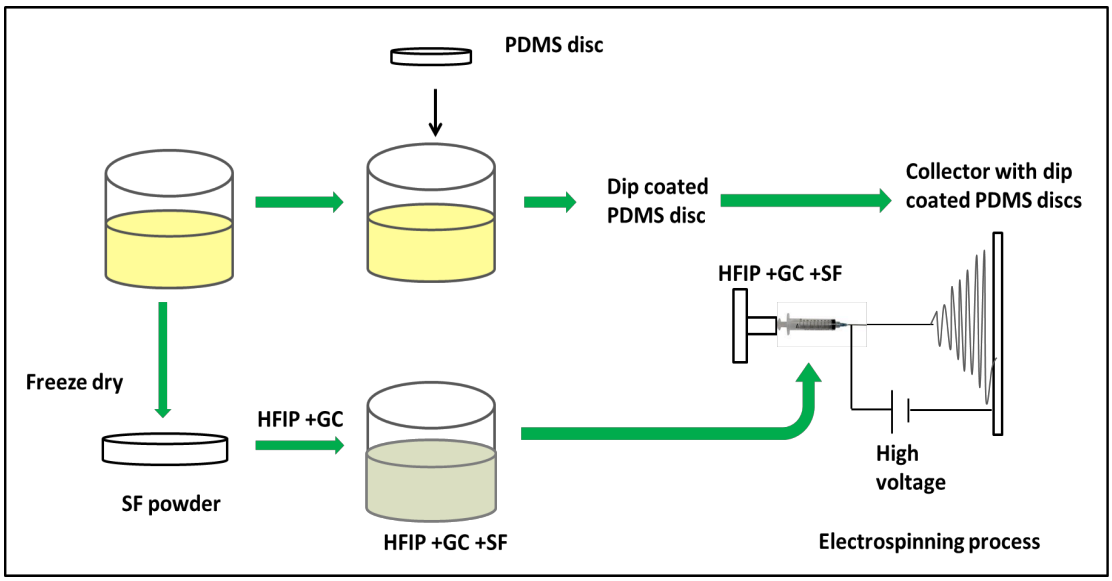


Figure 4a.1 Schematic representation of the process for SFGC-DES discs

4a.2.6 Scanning Electron Microscopy

The surface and cross-sectional morphology of the coated/uncoated PDMS discs was observed using a scanning electron microscope (SEM) - Quanta 200 3D from FEI. Representative images at appropriate magnifications were recorded after sputter coating the samples with a thin coating of gold to prevent sample charging.

4a.2.7 Atomic Force Microscopy

The surface topography of the uncoated/coated PDMS discs was performed in ambient conditions in tapping mode for a 40 µm x 40 µm area with a scanning probe microscope (JPK, NanoWizard II). Silicon cantilevers with a spring constant k of 60 N/m were used to perform experiments. The cantilever was oscillated at its first resonance frequency (250 - 350 kHz).

4a.2.8 Mechanical stability of the coating

The crack resistance of the coating was measured using a 180° bending test with a rectangular block of 1 cm x 3 cm x 0.1 cm (thickness) as per the protocol described by Borkner et.al. (2014)[23] The central portion of the coated & uncoated PDMS strip was imaged in the SEM.

4a.2.9 Contact angle measurement

The modification of PDMS and their coating was confirmed by 4 µl stable sessile drop of DI water. The equilibrium contact angle was reported as the average of at least four measurements for each modification.

4a.2.10 Fourier Transform Infrared (FTIR) spectroscopy

The uncoated and coated discs, before and after methanol treatment, were characterized using ATR-FTIR Perkin Elmer Q5000 Spectrum GX spectrophotometer equipped with a diamond crystal probe detector. The scan was recorded from 500 cm^{-1} to 4000 cm^{-1} with a resolution of 4 cm^{-1}.

4a.2.11 GC release from the coating

The GC release from the SFGC-DES discs was determined by keeping the coated discs immersed in Phosphate Buffered Saline (PBS) solution for seven days at 37 ^{o}C, and UV-vis spectroscopy was done on the PBS solution to measure the amount of GC released at predefined time points. The concentration of GC released into PBS was estimated by recording the absorbance at 208 nm^{-1}. The experiment was carried out in triplicates.

4a.2.12 Anti-quorum sensing assay of SF and GC coated PDMS discs

E. coli pSB1142 strain (a kind gift from Dr. Paul Williams, University of Nottingham), was used as a biosensor strain for detection of QSI activity. It was grown in Luria-Bertani (LB) medium supplemented with 10 µg/ml tetracycline (Sigma Aldrich, India) at 37 °C. SF-DES and SFGC-DES discs were dipped in PBS for 24 h for releasing the active compound (GC), and anti-QS activity of the released compound was checked using *E. coli* pSB1142 as per method described elsewhere[24]. The overnight grown culture of *E. coli* pSB1142 (OD_{600nm}: 0.1) was incubated along with GC released in PBS and 0.1 µg/ml of the signal molecule (3-oxo-C12-HSL)

(Sigma Aldrich, India). Reporter strain with and without signal molecule was considered as a positive and negative control respectively. All experimental sets were incubated at 37 °C at 180 rpm for 6 h. Luminescence was measured using microplate reader (Spectra Max MS^c molecular devices) and reported as the relative light unit (RLU) normalized with OD at 600 nm. Experiments were performed in triplicates and percentage of (RLU/OD_{600nm}) inhibition was calculated by using the formula:

$$\text{Luminescence inhibition } (RLU/OD_{600nm})\% = \frac{\text{Positive control } (RLU/OD_{600nm}) - \text{Test } (RLU/OD_{600nm})}{\text{Positive control } (RLU/OD_{600nm})} \times 100$$

4a.2.13 Anti-biofilm assay of SF and SF-GC coated PDMS discs

The anti-biofilm activity of the uncoated/coated PDMS disc was evaluated using *P. aeruginosa* (NCIM 5029) and *S. epidermidis* (NCIM 5270) as test organisms. Initially, overnight grown cultures of *P. aeruginosa* and *S. epidermidis* were subcultured to petri plates (30 mm diameter) containing 2 ml LB medium (OD_{600} nm was adjusted to 0.1). SF-DES and SFGC-DES PDMS discs were then added to these petriplates. After incubation of the plates for 24 h at 37 °C, biofilms formed on the discs were subjected to crystal violet staining. The stained biofilm was then visualized under a light microscope at 400x magnification[25]. Biofilm formed on the PDMS discs were also visualized by SEM. For quantitative evaluation of anti-biofilm activity, the crystal violet stain was extracted from the discs using 30 % acetic acid. OD of the extracted crystal violet was measured at 580 nm.

4a.2.14 *In-vitro* cell proliferation and cytotoxicity study:

In-vitro cell cytotoxicity of modified PDMS disc was studied by using L929-mouse fibroblast cell line (Purchased from NCCS, Pune, Maharashtra, India) [26]. L929 cells were seeded separately on PDMS, SF-DES, SFGC-DES discs for three different incubation times (1, 2, and 3 days) in a 96 well plate. $5x10^3$ cells/disc were used in 10 µl of DMEM (Invitrogen) supplemented with 10 % FBS (Invitrogen). All the plates were incubated with 5 % CO_2 at 37 ºC for 10 min that allows the cells to settle on the scaffold. After 10 min, 250µL of DMEM supplemented with 10 % FBS was added and further incubated. After each incubation, i.e. after 1st, 5th and 7th day, the media

was removed from each plate and filter sterilized MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (0.45 mg/ml) (Invitrogen) prepared in DMEM containing 10 % FBS and incubated for 4 h at 37ºC with 5 % CO_2 at 37 ºC. Further, MTT was removed from the well, and DMSO 200 µl/well was added to dissolve insoluble formazan crystals and incubated with 5 % CO_2 at 37 ºC for 10 min. The absorbance was measured at 550 nm using a microtitre plate reader (Multiskan EX, Thermo Scientific). Experiment was carried out in triplicates[27].

4a.3 Results and discussion:

4a.3.1 Characterization of the SFGC-DES coating

PDMS is a polymer routinely used in a variety of biomedical devices and implants. PDMS implants are frequently coated with functional molecules/polymers to improve biocompatibility, add lubricity or antimicrobial functionality to the surface. In our work we have used a novel crack resistant coating process patented by Nisal et al. (2017) which involves sequential dip coating and electrospinning technique[22].

Though dip coating of thin layers (<10 nm) of SF on quartz substrates has been demonstrated earlier[18], these coatings are too thin to achieve a sustained or prolonged release of a functional drug molecule and the researchers later proposed another method of using silk barrier layer to suppress the initial burst release of functional molecule[28]. But, they did not comment on the mechanical stability of the coating, However, when an elastomeric substrate like PDMS is coated with a thick layer of biomaterials like GC-SF, it is necessary to understand the effect of mechanical deformation of the substrate on the coating. Thus, to improve the stability of coatings under mechanical deformation, an innovative coating process was used here.

A combination of two conventional processing protocols - dip coating and electrospinning was combined to form a self-reinforced coating. (Sample abbreviation: SF-GCDES). The coating was first characterized using SEM, and the results have been summarized in Figure 4a.2.

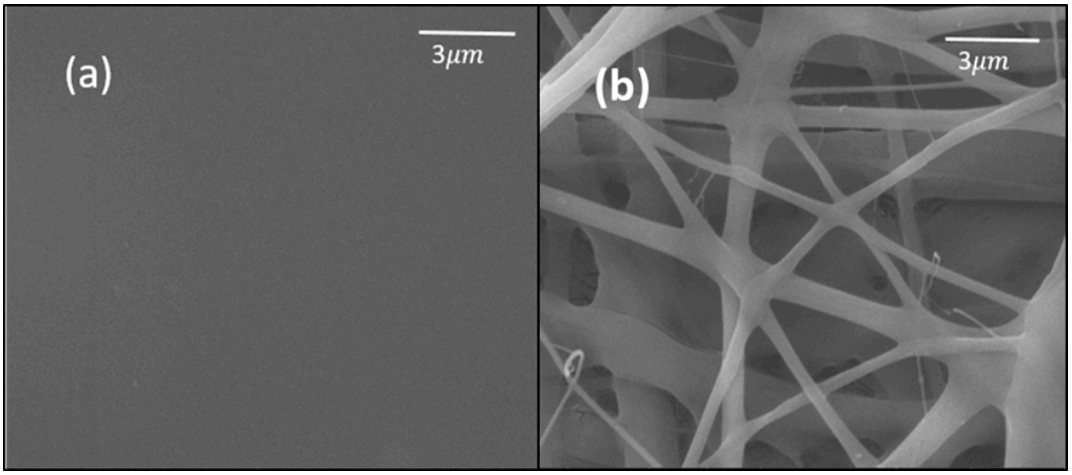


Figure 4a.2: The SEM images of PDMS coatings: a) only PDMS b) SFGC-DES

Distribution of fiber diameter of GCSF electrospun fiber was determined using Image J software by measuring fiber diameter of 50 different locations from Figure 4a.3 (a). Results showed that 80 % electrospun fibers were having diameter in the range of 200-400 (Figure 4a.3 (b)). The electrospun fibers are partially embedded in the dip coated SF matrix and some fibers also appear to be on the surface of the coat as can be seen in Figure 4a.2 (b); and Figure 4a.3 (a)

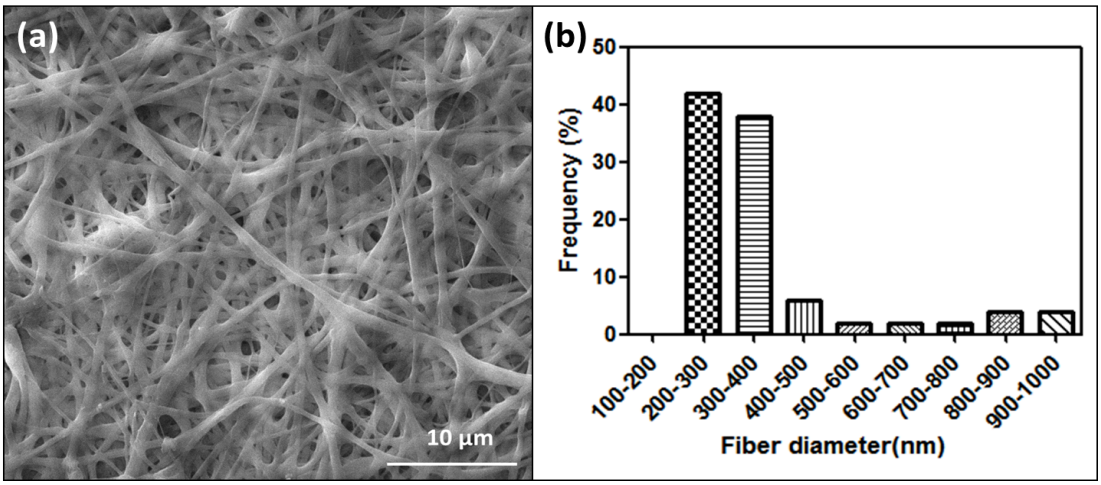


Figure 4a.3 Distribution of fiber diameter of GC-SF electrospun fibers: (a) SEM image of PDMS coating SFGC-DES (b) Graph showing distribution of fiber diameter of GC-SF electrospun fibers: 42 % fibers were having diameter in the range of 200-300 nm however, 38 % fibers showed diameter in the range of 300-400 nm.

The surface topography of uncoated/coated PDMS discs was studied using atomic force microscopy in tapping mode, and these results are summarized in Figure 4a.4. The disc with sequential dip and electrospun GC SF exhibits a surface roughness that is at least an order of magnitude higher than that of only PDMS disc (Figure 4a.5). This increased surface roughness is desirable for biomedical applications. It has been shown that the fibroblast attachment on textured breast implants was higher than smooth implants by Valencia-Lazcano et al. (2014)[29]. Researchers have also demonstrated that proliferation of fibroblast was better in electrospun mats as compared to SF films[30,31].

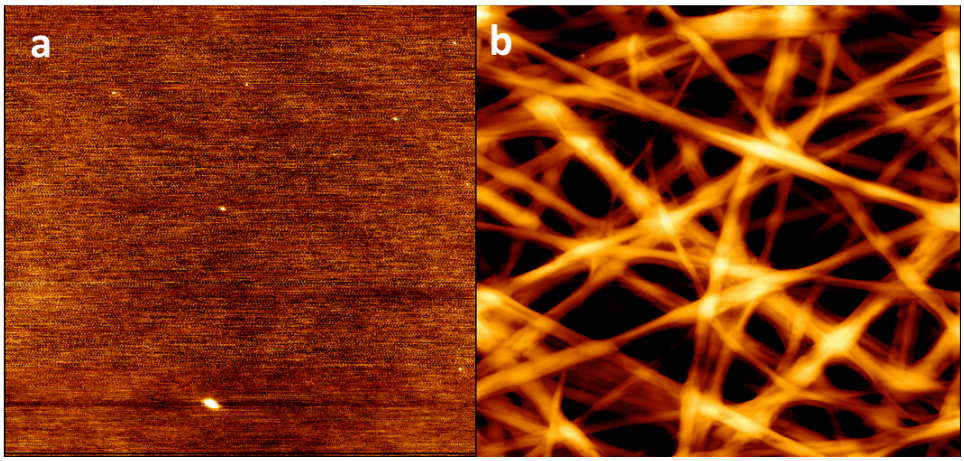


Figure 4a.4: Atomic force microscopy of PDMS coating (a) only PDMS disc (b) SFGC-DES disc

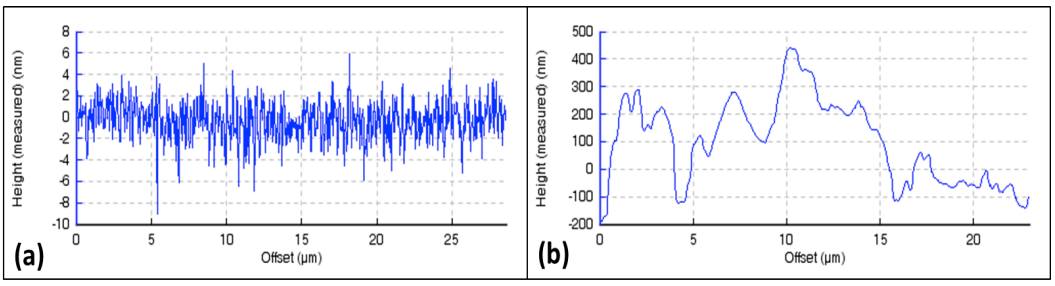


Figure 4a.5 Height profile measurements of (a) PDMS disc without coating (b) SFGC-DES coating on PDMS. The surface topography of PDMS before and after coating with GC-SF shows surface roughness of 2 nm, 119 nm respectievly.

4a.3.2 Analysis of mechanical stability of the coating

As per prior literature recombinant spider silk coating on PDMS caused cracking of coating after bending test[32]. Therefore, a simple bending test, followed by SEM imaging, was designed to characterize the cracking resistance of the coating on the PDMS implant. As can be seen from the Figure 4a.6 (a)(c), the PDMS disc surface without coating did not exhibit any changes in surface morphology after the bending test. Similarly, the surface morphology of the novel dip + GC SF Espun PDMS disc surface was comparable before and after the bending test as can be seen in Figure 4a.6 (b)(d). The GC SF electrospun fibers exhibit regions of stress whitening indicating that the mechanical stress exerted on the samples is primarily taken up by the fibers and thus, the damage caused to the lower dip coated layer is minimum. Also, as discussed earlier, the electrospun nanofibers are partially embedded in the dip-coated layer. It may, therefore, be hypothesized that these embedded fibers act as reinforcement and improve the cracking resistance of the coating. Self-reinforcement of polymer matrices using nano/microfibers is a classical and proven technique to improve the toughness of the material[33–35].

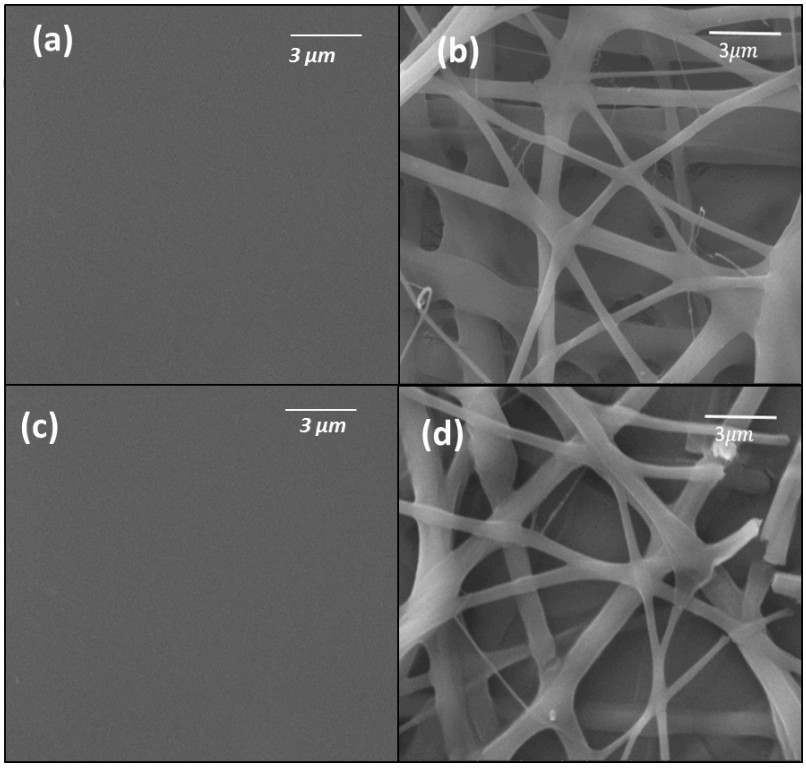


Figure 4a.6: Scanning Electron Micrographs of the coated PDMS discs before and after bending test: (a), (b): PDMS discs without coating and SFGC-DES respectively

before bending (c), (d) PDMS disc without coating and SFGC-DES respectively after bending.

Thus, our results demonstrate that this novel method significantly enhances the performance of the coating. This performance improvement is highly desirable considering that implants are subjected to a variety of mechanical stresses both during handling and in use.

4a.3.3. Contact angle of PDMS surface after SFGC-DES coating

The PDMS surface was characterized after GC-SF electrospinning using a simple instantaneous contact angle measurement to confirm successful modification. The pristine PDMS polymer surface is highly inert and unreactive, and hence it is extremely challenging to have a coating adhere to the PDMS surface. The SFGC-DES discs had a contact angle of 85 ± 5^{o}, which is much lower than the PDMS disc without any coating (115 ± 5^{o}). GC is a hydrophilic molecule and hence improves the wettability of a surface (Figure 4a.7).

Thus, the change in contact angle after coating with GC-SF provides sufficient evidence to corroborate the modification of the PDMS surface and its successful coating.

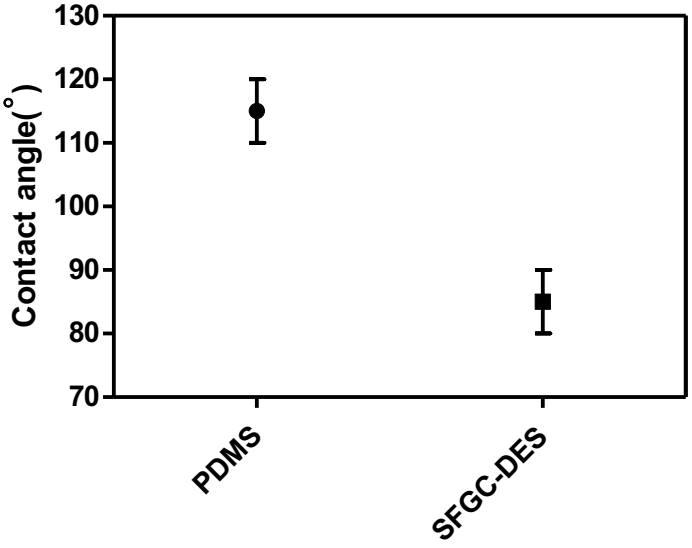


Figure 4a.7: Contact angle measurement on coated (SFGC-DES) /uncoated PDMS discs

4a.3.4. Analysis of Fourier Transform Infrared (FTIR) Spectroscopy

The modification of the PDMS substrate after coating was also characterized using ATR-FTIR spectroscopy to gain insights into the conformation of the SF protein. The control samples used here were pure PDMS substrate, SF degummed fibers and as synthesized GC molecule. The pristine PDMS has no signal beyond the 3000 cm^{-1} range. The symmetric and asymmetric streching of CH_3 shows peaks at 2963 cm^{-1} and 2906 cm^{-1}, along with CH_3 bending vibration at 1258 cm^{-1}. The peak in the range 1200 cm^{-1} to 800 cm^{-1} confirms the presence of Si-O-Si groups of PDMS[36]. G-citron shows a peak at 3063 cm^{-1} to 3640 cm^{-1} corresponds to –OH streching from glucose. The asymmetric and symmetric stretching of CH_2 gives a peak at 2857 $cm^{-1.}$ The presence of C=O stretching in glucose gives a peak at 1720 cm^{-1} due to saturated aliphatic 6 membered ring of glucose (Figure 4a.8). The random coil conformation of silk fibroin shows a peak at 1650 cm^{-1} and 1540 cm^{-1} for amide I and amide II peak respectievly[21]. The SF-GC coated PDMS exhibited peaks from both pure SF and the PDMS substrate. This is anticipated since the IR beam is known to penetrate the first few microns of the surface. Since the coating is only ~1 μm in thickness, the spectrum also shows peaks corresponding to the below PDMS substrate. The Amide I stretching peak at 1623 cm^{-1} indicates that the protein has predominantly beta sheet conformation. Earlier reports suggest that SF after electrospinning displays lower beta sheet content. However, the methanol vapor post treatment results in the change in protein conformation. The IR spectrum displays a prominent Amide I peak at the lower wavenumber of 1623 cm^{-1}. Conversely, the GC peaks in SFGC-DES were too weak to be discerned and quantified (Figure 4a.8).

Hence, the presence of GC in the coatings was confirmed by UV-vis Spectroscopy of the supernatant solution of SFGC-DES after incubated at 37 ^{o}C for 24 h. The peak at 208 nm confirmed that GC was released from the coating (Figure 4a.9). Thus, in addition to the change in contact angle observed between the PDMS without any coating and SFGC-DES sample, GC release from the coatings can be considered to be proof of successful incorporation of GC in the coatings.

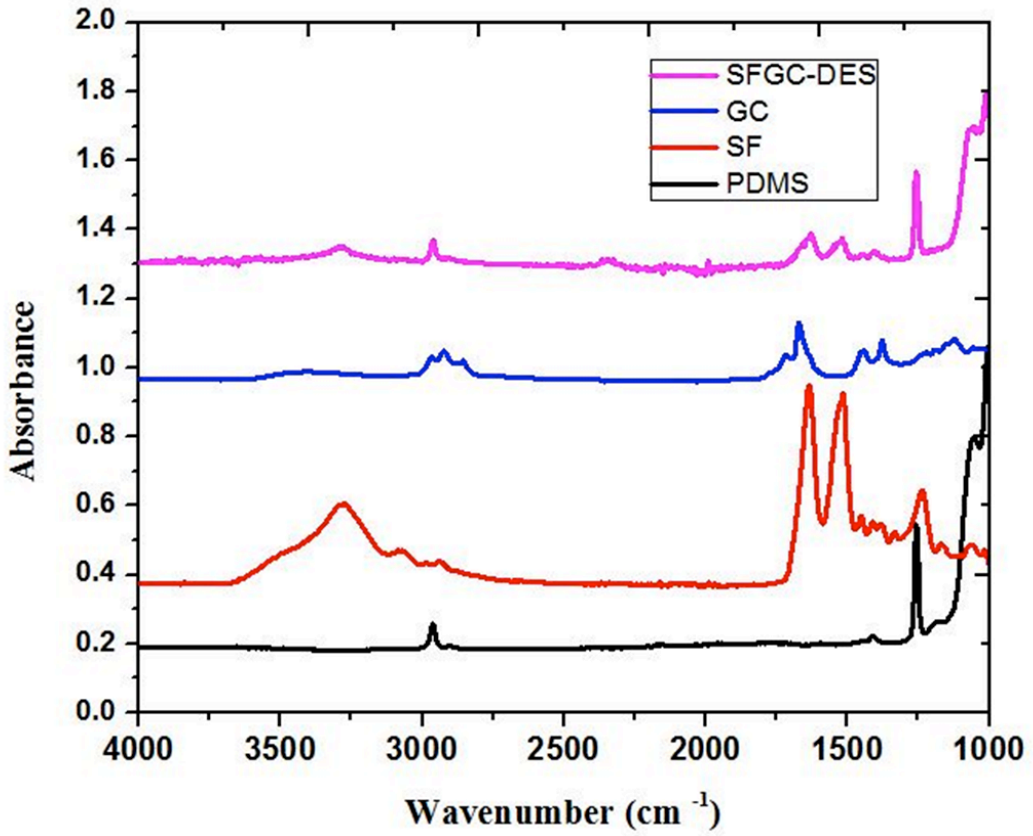


Figure 4a.8: FTIR spectra of SFGC-DES, GC, SF, and PDMS

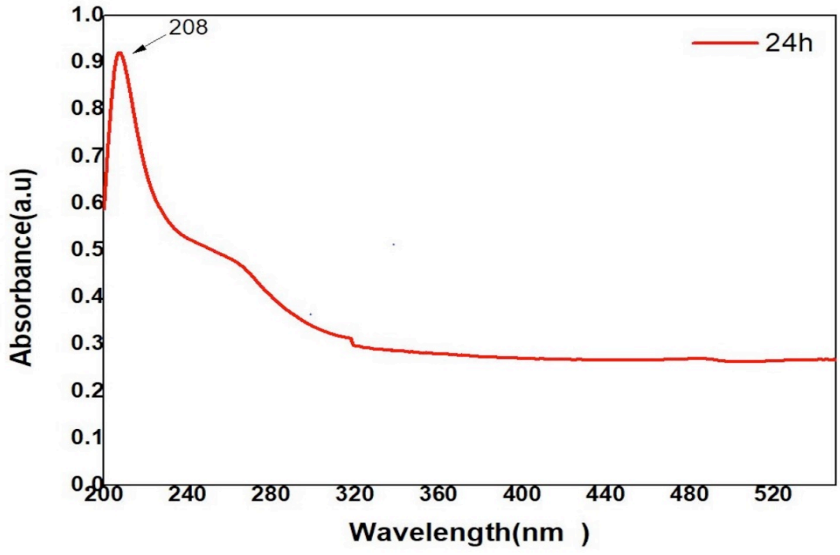


Figure 4a.9: UV-vis Spectroscopy of the supernatant solution of SFGC-DES after incubated 37 °C for 24 h : The SFGC-DES discs were incubated in PBS (neutral buffer) at 37 °C for 24 h; The supernatant solution was used for UV-vis Spectroscopy.

The absorbance was measured at 208 nm confirms that GC was released from the coating

4a.3.5 GC release profile of the SFGC-DES coating

The GC released from the SFGC-DES coating was determined using UV-vis spectroscopy. SFGC-DES discs were incubated in PBS solution for 7 days at 37 °C and the release of GC into the PBS solution was monitored using UV-vis spectroscopy and the data has been summarized in Figure 4a.10. The GC in aqueous solutions shows absorbance at 208 nm^{-1} and this absorbance was used to estimate the amount of GC released from the coating. As can be seen from Figure 4a.10, the amount of GC released from the coating after immersing in PBS showed sustained release till 7^{th} day. Thus, it may be concluded here that the SF helps in sustained release of GC and therefore SFGC-DES coating can be used in biomedical application where sustained release of active compound is desired.

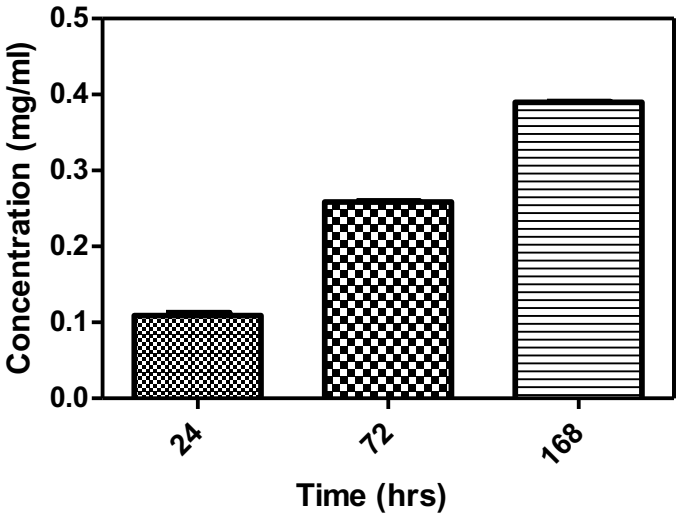


Figure 4a.10: Graph of GC release profile till 7^{th} day

After completion of the mechanical and physicochemical characterization of the coatings, the coatings were further evaluated for their biological activity. As discussed earlier, one of the primary reasons for the failure of implants is the formation of a bacterial biofilm. Also, the growth of microorganisms in biofilms shows high resistance to antimicrobial agents. This is probably due to decreased penetration of antibiotics and decreased growth rate or metabolism of the bacterial cells present in the biofilm. Antibiotic gives selective pressure to bacteria, and thus,

they indeed find ways of resisting the newly developed antibiotics[37]. High doses of antibiotic are required to be administered, which in turn affects the normal flora of the host body as well. Quorum sensing inhibition provides a promising alternative to antibiotic resistance by altering bacterial communication, without putting selective pressure on bacteria as well by inhibiting biofilm formation[38]. It is a means of communication between the bacterial cells using specific diffusible signal molecules called as auto-inducers specific to their species. These molecules aid bacteria to sense their population density and to regulate the expression of various genes in response to the density[39]. Quorum sensing controls the expression of genes for virulence, motility and biofilm formation, etc. which play a crucial role in the pathogenesis[38]. Thus, molecules, which target QS in the bacterial population, automatically also, inhibit biofilm formation, the resultant of QS.

Reports suggest that plant-derived essential oils act as quorum sensing inhibitors[25]. But their biological activity cannot be exploited fully due to their hydrophobicity. It has already been demonstrated in earlier chapters that GC (hydrophilic derivative of citronellal) exhibit excellent antibiofilm and quorum quenching activity. Therefore, antiquorum sensing potential of GC after coating on PDMS was confirmed.

4a.3.6 Antiquorum sensing activity of SF and GC coated PDMS discs

E. coli pSB1142 was used to check the inhibition of quorum sensing mediated through long-chain AHL signal molecule. The strain possesses lasR and las promoter of *P. aeruginosa* fused to the luxCDABE cassette from *Photorhabdus luminescens* which responds to long-chain AHLs (C10-C14) to produce luminescence. This reporter strain does not synthesize AHL molecules but exhibits luminescence in the presence of external AHL signal molecule[40]. In this experiment, the external signal molecule selected was N-(3-oxo-dodecanoyl)-L-homoserine lactone, one of the signal molecules produced by *P. aeruginosa*. The decrease in the luminescence of the cells (compared to positive controls) in the presence of coating confirms its anti-QS activity. To exclude growth-dependent effects, OD measurements were recorded in order to normalize luminescence production to cell density. Using PDMS discs with and without coating, QSI activity of the released compound was checked when the 3-oxo-C12 HSL signal molecule was added externally. Active compund released from

disc coatings with GC SF showed significant quorum sensing inhibition which is more than 50 % when compared to PDMS discs without coatings (Figure 4a.11).

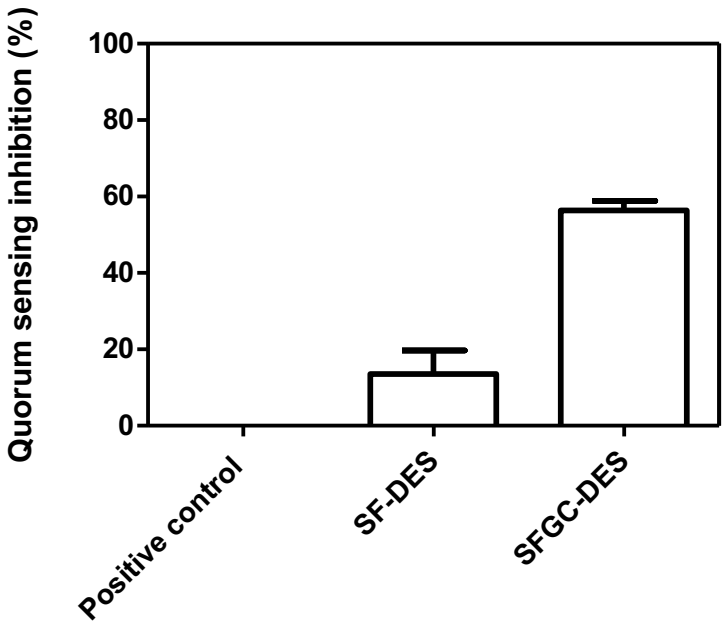
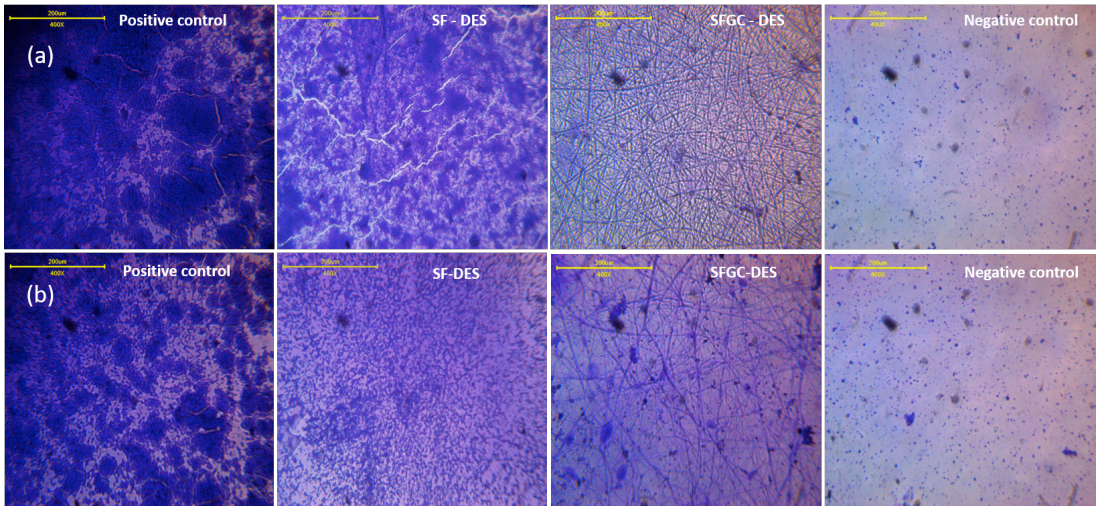
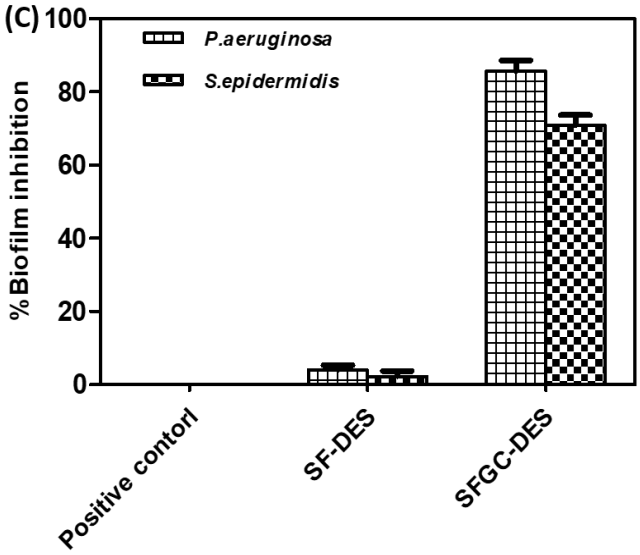


Figure 4a.11: Anti-quorum sensing activity of coated and uncoated PDMS discs

4a3.7 Antibiofilm activity of SF and GC coated PDMS disc

The formation of bacterial biofilms is one of the prime phenomenon controlled by quorum sensing. Impeding capacity of bacteria to form biofilm is a major challenge in treating chronic infections. In this study, the antibiofilm activity of SF-DES and SFGC-DES PDMS disc was evaluated by using *P. aeruginosa* (NCIM 5029), *S. epidermidis (*NCIM 5270) as test organisms. The PDMS disc containing GC in the coating showed significant antibiofilm activity when observed after 24h. Around 85 % biofilm inhibition when tested against *P. aeruginosa* was observed whereas the same coating showed nearly 70 % biofilm inhibition when tested against *S. epidermidis*. These results have been summarized in Figure 4a.12 a, b, c. Higher magnification scanning electron micrographs also support our observations (Figure 4a.12 d, e).


(a)
Positive control
SF - DES
SFGC - DES
Negative control
(b)
Positive control
SF-DES
SFGC-DES
Negative control


(C)
% Biofilm inhibition
100
80
60
40
20
0
P.aeruginosa
S.epidermidis
Positive contorl
SF-DES
SFGC-DES

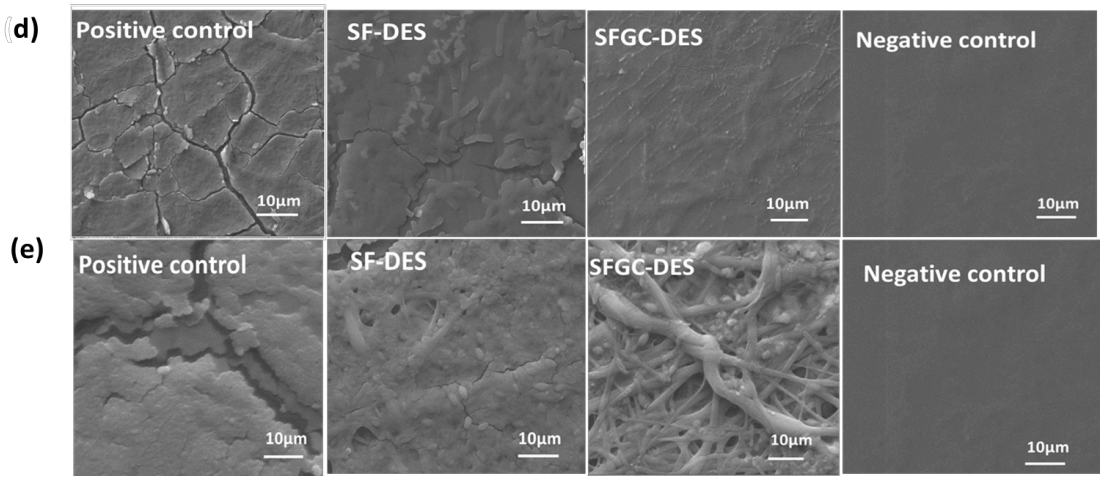

(d)
Positive control
SF-DES
SFGC-DES
Negative control
10µm
(e)
Positive control
SF-DES
SFGC-DES
Negative control
10µm

Figure 4a.12: Light microscopic and SEM images of biofilm developed on SFGC-DES and SF-DES coatings and biofilm quantification: Optical microscopy images of biofilm inhibition observed on coated and uncoated PDMS discs stained with crystal violet (a) *P. aeruginosa* b) *S. epidermidis*

(c) *S. epidermidis* and *P. aeruginosa* biofilm inhibition quantified by extracting crystal violet stain from the coated/uncoated PDMS discs.

SEM images of biofilm inhibition observed on coated and uncoated PDMS discs (d) *P. aeruginosa* (e) *S. epidermidis*

4a.3.8 *In-vitro* cell proliferation and cytotoxicity of SF and GC coated PDMS discs

The coated and uncoated PDMS discs were evaluated for cytotoxicity using *in-vitro* cell culture studies with mouse fibroblast cell line L929. Cell proliferation was evaluated by performing the MTT test. MTT data in Figure 4a.13 shows increase in absorbance on all days in SF-DES and SFGC-DES as compare to PDMS that indicates greater biocompatibility was achieved with electro-spinning technique. Higher cell viability among all three groups was observed in SFGC-DES discs on day 1, confirming the non-cytotoxicity of GC. Similar cell viability trend was observed on 5th and 7th day that is sufficient to prove the biocompatibility of the modified discs.

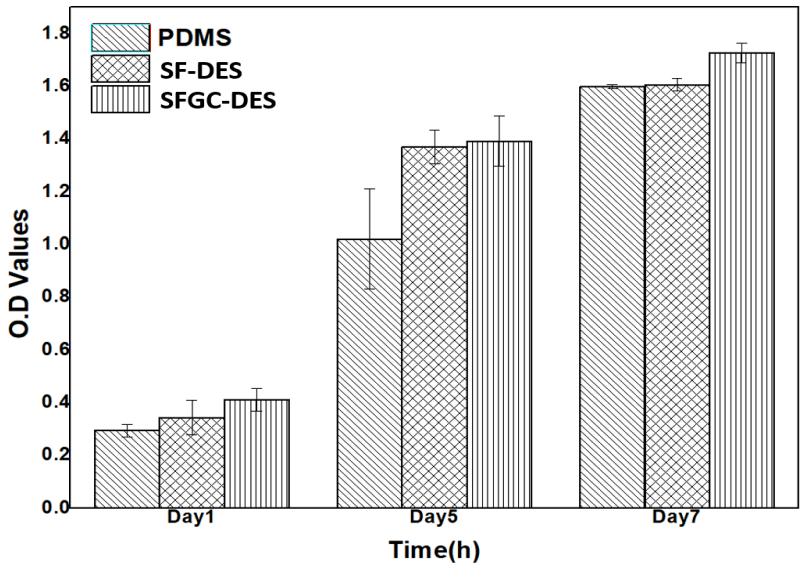


Figure 4a.13: MTT Assay using L929 cells on PDMS, SF-DES and SFGC-DES samples for 7 days

4a.4 Conclusion

Surface coatings have emerged as a promising alternative to reduce implant failure due to bacterial infection, foreign body response and mechanical deformation. In this chapter, we have used novel process innovation – sequential dip and electrospinning process, that results in the formation of the self-reinforced coating made up of next generation quorum quenching antibiofilm molecule GC and SF (a natural biocompatible protein polymer) on an elastomeric substrate like Polydimethylsiloxane. GC molecule not only retains its quorum sensing activity throughout the processing steps but also inhibits biofilm formation of two of the most notorious infection-causing bacterium - *P. aeruginosa* and *S epidermidis* thus will inhibit implant failure due to bacterial biofilm formation. These new coating has shown to be crack resistant under mechanical deformations in bending modes. GC and silk fibroin improves the biocompatibility of the PDMS substrate thus, mitigating the risk of implant failure due to foreign body response. Thus our work demonstrates an innovative solution to simultaneously resolve multiple causes for failures of implant devices – biofilm formation, foreign body response and cracking of the coating.

This chapter has been published as:

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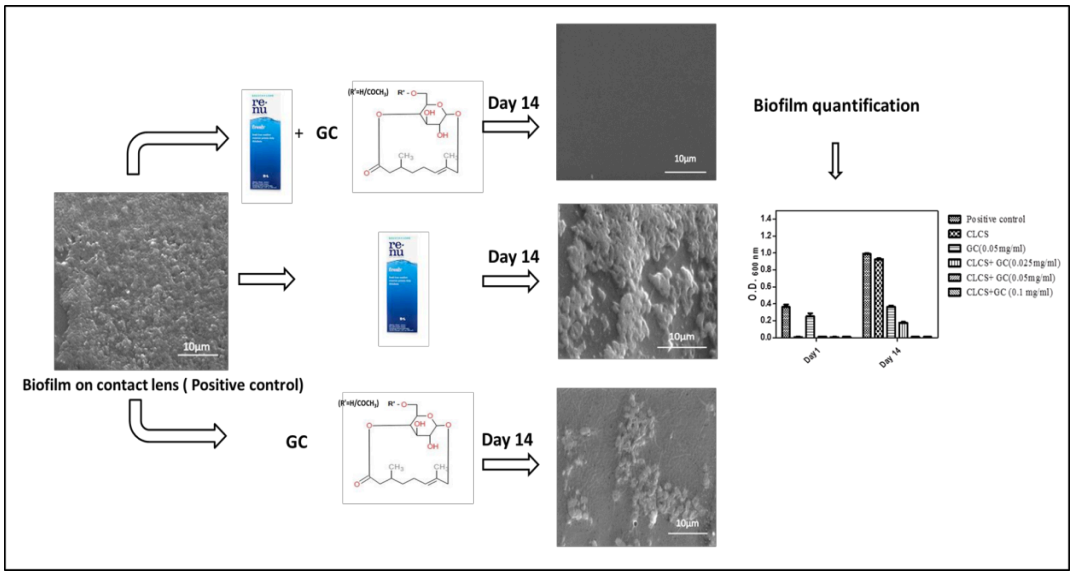
Chapter 4b

Synergistic effect of G-citron and lens cleaning solution to increase the antibiofilm effect



Although contact lenses (CL) have made a tremendous improvement in the eye care field, CL wearers are most susceptible to eye infections. More than 50 % of CL linked corneal infections are mainly caused due to bacterial colonization and deposition of bacterial toxins and byproducts on contact lenses. Among all contact lens-associated infections microbial keratitis(MK), contact lens-induced peripheral ulceration(CLPU), infiltrative keratitis (IK), microbial colonization of contact lenses include contact lens-induced acute red eye (CLARE), and asymptomatic infiltrative keratitis (AIK) are reported very frequently. Gram-negative bacteria are the primary causative agent of CL associated microbial keratitis and among them *Pseudomonas* spp. are the most commonly found organism in this infection. It has been also reported that quorum sensing mechanism present in *P. aeruginosa* trigger CL linked

infections by initiating biofilm formation, virulence factor production like protease, pyoverdine.

Therefore in this chapter, we have shown another application GC (novel QSI molecule; synthesis and QSI activity mentioned in chapter 2a, 2b) in contact lens cleaning solution to reduce biofilm formation on the contact lens. We have demonstrated that the GC retained its quorum sensing inhibitory activity when used along with "renu advanced formula multi-purpose solution" and improved the antibiofilm efficacy of contact lens cleaning solution.

4b.1.Introduction

Human's eyes have got privileged due to many factors which prevent bacterial infections. However, cases of eye infections have increased due to an external entity like contact lenses. Though in the ocular field contact lenses (CL) have got more importance as it helps to improve vision, contact lens wearers are at highest risk of the eye infections[1]. 60 % of CL associated corneal infections are of bacterial origin[2]. Several bacteria colonize CL and cause various infections but Gram-negative bacterium *P. aeruginosa* is one of the predominant bacteria involved in eye inflammation conditions and infections. *P. aeruginosa* is pre-dominantly isolated and major etiological agent of severe corneal infection called as microbial keratitis (MK)[3]. Approximately 50 % of MK cases are associated with contact lens practitioners[4]. If it is not treated early and with proper medication destroys cornea and leads to blindness. MK may also become severe due to excessive activation of host defense system. Corneal epithelial cells produce inflammatory response mediators such as cytokines and chemokines on account of *P. aeruginosa* recognition by their toll-like receptors (TLRs). These mediators continuously recruit polymorphonuclear neutrophils and white blood cells to the site of infection leading to the destruction of corneal cells and tissue components[5]. It has also been reported that quorum sensing ability of *P. aeruginosa* plays a vital role in the progression of MK. Quorum sensing mediates bacterial colonization, virulence factor production, protease synthesis which helps to either invade or kill corneal cells. Other adverse consequences directly linked to bacterial colonization include contact lens-induced peripheral ulceration (CLPU), infiltrative keratitis (IK), microbial colonization of contact lenses include contact lens-induced acute red eye (CLARE) and asymptomatic infiltrative keratitis (AIK)[5].

IK and AIK are mainly triggered by the presence of bacterial toxins, enzymes, and byproducts on lens surface[6]. However, bacteriologic examination of CLARE and CLPU condition is mainly attributed to presence *P. aeruginosa* and *Staphylococci spp*. respectively[2].Contact lens cleaning solutions (CLCS) usually plays major role in reducing above mentioned contact lens associated microbial infections. As these infections are mainly occurred because of bacterial adhesion and biofilm formation, CLCS should inhibit biofilm formation on contact lenses[3]. Though, currently available contact lens cleaning solutions help to reduce CL associated eye infections, the occurrence of infections not yet reduced completely. The major reason behind this failure could be quorum sensing ability of pathogenic organism which supports virulence factor production and biofilm formation on the contact lens. Antibiotics impose selective pressure on the pathogenic bacteria and increasing the burden of antibiotic resistance. In such scenario, using quorum sensing inhibitors (QSI) in contact lens cleaning solution (CLCS) could be a novel approach, however, effect of QSI molecule on *P. aeruginosa* biofilm development on contact lens has not been quantified and studied before.

Therefore in the current chapter we have demonstrated that the efficacy of "renu multi-purpose solution", one of the popular contact lens cleaning solutions currently available in the market can be improved in terms of its antibiofilm effect using novel G-citron (GC) molecule (synthesis and QSI activities of molecule have been mentioned in Chapter 2a and 2b). Thus, the main objective of the work was to explore the application of the GC to prevent contact lens-associated infections.

4b.2 Materials and methods

4b.2.1 Bacterial strains and other materials:

P. aeruginosa NCIM 5029 was grown in LB broth at 37 °C. Renu multi-purpose solution, and contact lenses made up of high water (>50 % H_2O) content, and the nonionic polymer was chosen for our study.

4b.2.2 Assessment of the biofilm inhibition by the spectrophotometric method:

Contact lenses were incubated along with GC and CLCS individually and together for two weeks. Experimental additions were done as given in Table 4b.1. These experimental sets were incubated at room temperature for 1 day, 7 days and 14 days. In 5th set seven GC concentrations were checked (from 0.01 mg/ml to 0.25 mg/ml). The whole experiment was done in triplicates.

On first, seventh, and fourteenth day, contact lenses incubated as above were removed from the plate and gently washed in phosphate buffer saline (PBS), and then transferred to a new plate containing fresh LB media for re-culturing and incubated for 24 h. After incubation, the optical density (OD) of bacterial solutions was measured at a wavelength of 600 nm using a spectrophotometer[7]. We initially carried out direct staining and biofilm quantification on contact lenses using crystal violet after complete drying of the contact lens. But, we found that the contact lens itself was getting heavily stained by crystal violet. Therefore we used the indirect method for the quantification of biofilm by re-culturing from the biofilm, instead of direct quantification of biofilm.

Table 4b.1: Experiment sets designed for assessing biofilm inhibition on contact lenses.

		LB Medium (2ml)	***P. aeruginosa* (O.D.600nm: 0.1)**	**Contact lens cleaning solution (2ml)**	**G-citron (0.05mg/ml)**	**Saline (2 ml)**
1	Negative control	✓				✓
2.	Positive control	✓	✓			✓
3.	CLCS control	✓	✓	✓		
4.	GC control	✓	✓		✓	✓
5	CLCS +GC	✓	✓	✓	✓	

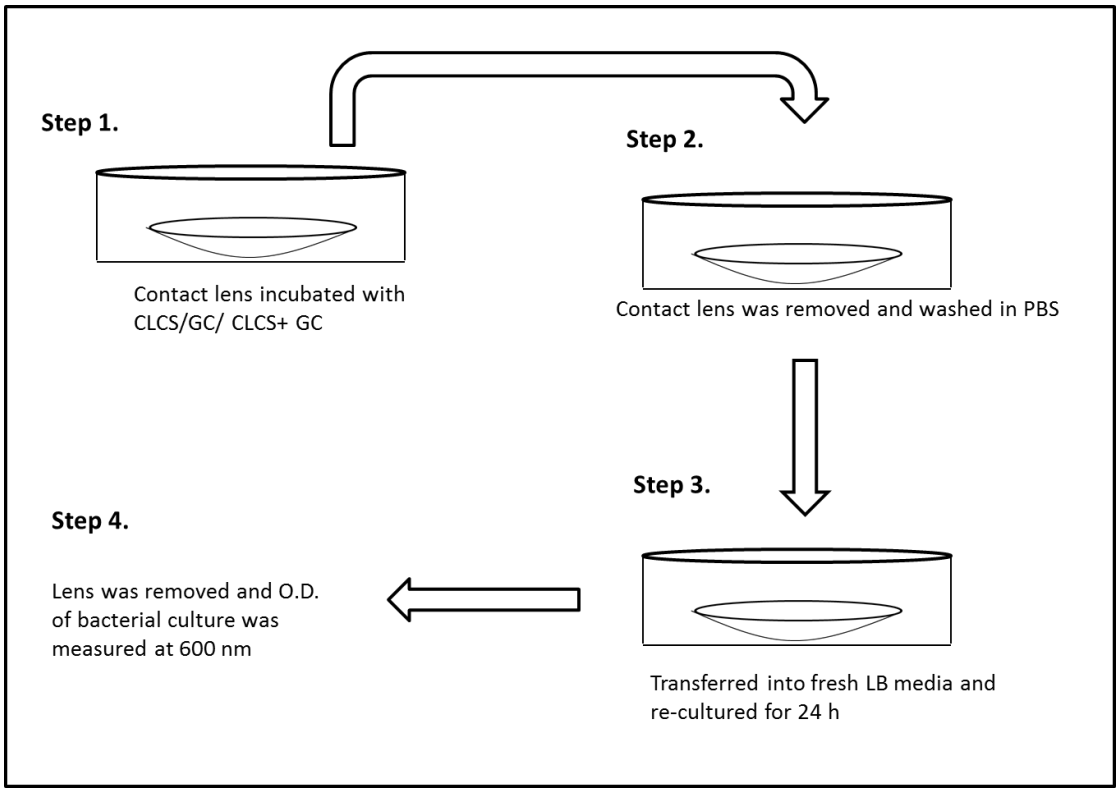


Figure 4b.1: Schematic representation of steps followed for quantification of biofilm developed on the contact lens in each experimental set mentioned in Table 4b.1.Biofilm developed on the contact lens is directly proportional to bacterial growth formed after re-culturing into fresh media.

4b.2.3 Scanning electron microscopy of the biofilm developed on contact lens

The surface of the contact lenses removed after 1st, 7th, 14th-day incubation from 5 different sets mentioned above was observed using the environmental scanning electron microscope (ESEM) - Quanta 200 3D from FEI. Representative images at appropriate magnifications were recorded after sputter coating the samples with a thin coat of gold to prevent sample charging.

4b.2.4 Assessment of the anti-adhesion capacity of GC:

Five concentrations of G-citron (1, 1.5, 2, 2.5, 3 mg/ml) were prepared in petri plates, and coverslips were kept in each plate. All coverslips were incubated at 37 °C for 24 h and then transferred to a fresh plate containing *P. aeruginosa* culture in 2 ml LB (final $O.D._{600nm}$= 0.1) and again incubated at 37 °C for 24 h. After incubation, biofilms formed on control (without prior incubation in GC solutions) and test (prior incubated in GC solutions) coverslips were visualized by staining with crystal violet.

The stained biofilm was then visualized under a light microscope at ×400 magnification[8,9]. Biofilm inhibition was evaluated quantitatively by extracting crystal violet stain of biofilm by using 30 % acetic acid. O.D. was measured at 580 nm.

To estimate concentration of GC on each dip coated coverslip, the coverslip was incubated in distilled water for 24 h and, the supernatant solution was used for UV-vis Spectroscopy. The absorbance was measured at 208 nm, and tentative concentration of GC was determined based on standard curve of GC.

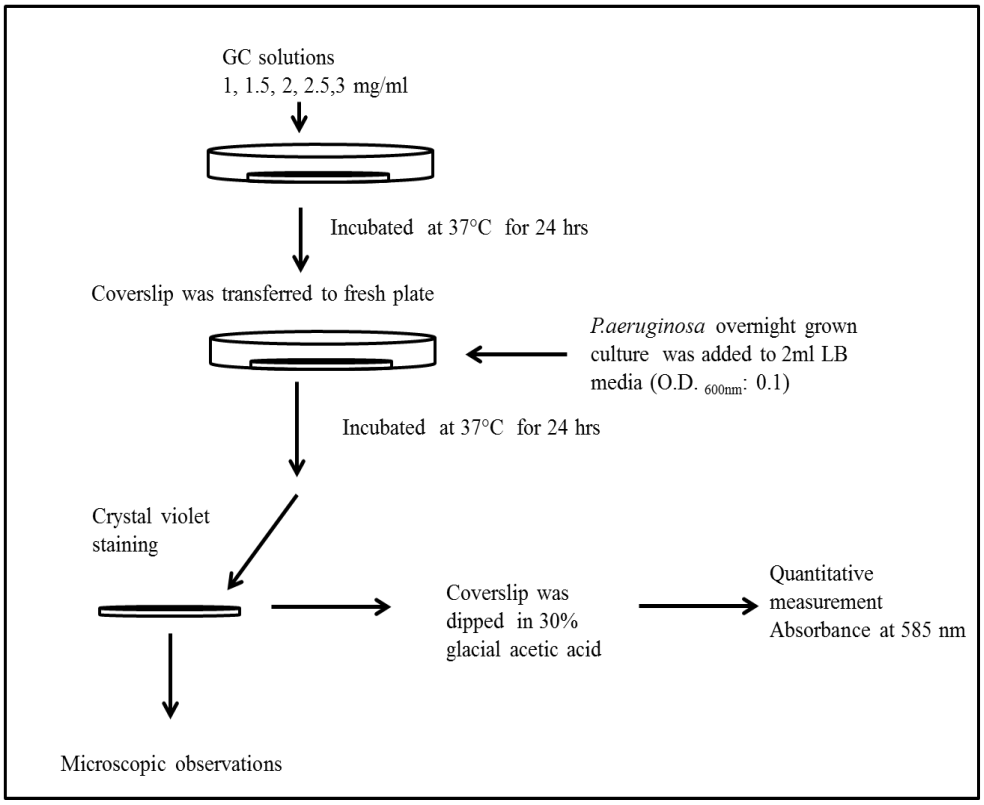


Figure 4b.2: Steps followed to assess the anti-adhesive potential of G-citron

4b.2.5. *In-vitro* cell proliferation and cytotoxicity study of GC:

GC cytotoxicity was evaluated using *in-vitro* cell culture studies with mouse fibroblast cell line L929 and rat muscle cell line L6. Concentration-dependent effect of G-citron on normal cell lines was investigated by MTT assay. It is the enzyme-based assay that determines mitochondrial dehydrogenase activity in the metabolically active living cells. In this assay, MTT, a yellow tetrazolium dye (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) is reduced into an insoluble dark purple color formazan product, by the action of NADPH dependent

mitochondrial reductase enzyme. The formazan crystals are then solubilized using an organic solvent (DMSO), and the absorbance is measured spectrophotometrically.

Cells with a density of $1x10^5$ cells/ml were seeded in DMEM media supplemented with 10 % fetal calf serum and grown overnight on a 96-well plate. After 24 h incubation, cells were treated individually with increasing concentrations of G-citron (5, 10, 20, 30, 40, 50, 100, 150, 200 µg/ml final concentration) at 37 °C with 5 % CO_2 for 24 h. Media was removed from all the wells and then 20 µl MTT (5 mg/ml) concentration was added to each well and incubated for 4 h at 37 °C. The cells were then treated with DMSO to solubilize the formazan crystals formed in viable cells and measured spectrophotometrically. Optical density was measured at 570 nm using SPECTRAmax plate reader (Molecular Devices Inc, USA) and the percent inhibition was calculated by using following formula[10]. All experiments were conducted in triplicates.

$$\text{Cell survival (\%)} = 100 - \frac{(Control\ OD_{570\ nm} - Test\ OD_{570\ nm}) \times 100}{Control\ OD_{570\ nm}}$$

4b.3. Results and discussion:

4b.3.1. Biofilm inhibition activity of GC:

GC (0.05 mg/ml) and CLCS synergistically inhibited *P. aeruginosa* biofilm formation on contact lenses after 7th and 14th day of incubation (Figure 4b.3, 4b.5) and was quantified by subsequent re-culturing of the contact lens in the fresh LB media for 24 h (Figure 4b.4). However, CLCS control did not show biofilm inhibition after 7th and 14th day of incubation which was observed under SEM (Figure 4b.5). This was also in accordance with quantification data of that set.

Conversely, though GC control showed turbidity in the incubated solution after the 14th day, this set showed nearly 70 % growth inhibition w.r.t. positive control in the subsequent re-culturing for 24 h which was clearly observed in SEM data of that set (Figure 4b.3, 4b.4, 4b.5). This indicated that GC must be inhibiting bacterial adhesion to the contact lens in addition to quorum sensing inhibitory activity.

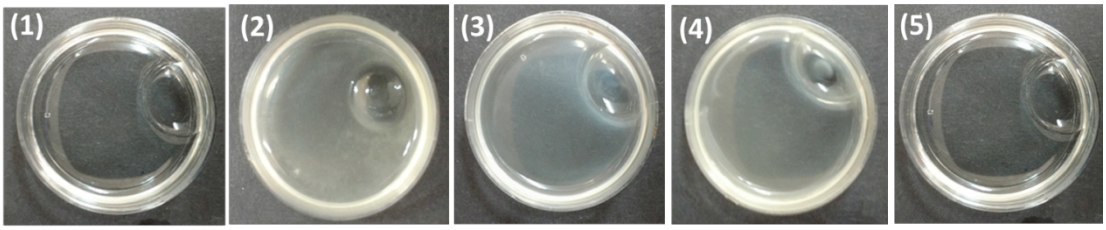


Figure 4b.3 Contact lenses incubated for 14 days: **(1)** Negative control : contact lens incubated in LB media (2 ml) and saline (2 ml) **(2)** Positive control: contact lens incubated in the presence of *P. aeruginosa* (OD_{600nm} : 0.1) in LB media (2 ml) and saline (2 ml) **(3)** CLCS control: contact lens incubated in the presence of *P. aeruginosa* (OD_{600nm} : 0.1) in LB media (2 ml) and CLCS (2 ml) **(4)** GC control: contact lens incubated in the presence of *P. aeruginosa* (OD_{600nm} : 0.1) in LB media (2 ml) and GC (0.05 mg/ml) in saline (2 ml) **(5)** contact lens incubated in the presence of *P. aeruginosa* (OD_{600nm} : 0.1) in LB media (2 ml) and GC (0.05 mg/ml) in CLCS

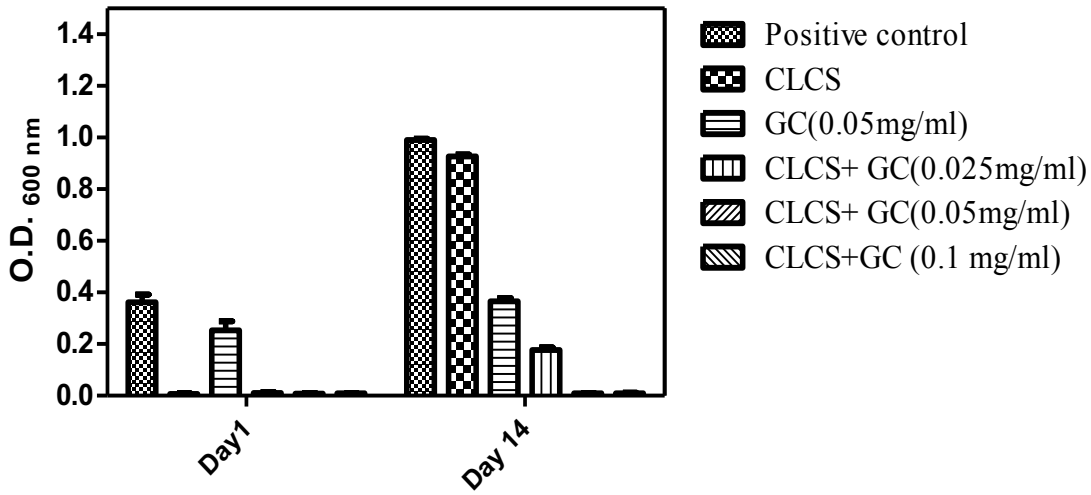


Figure 4b.4: Quantification of biofilm developed on contact lenses: After 1st day, 14th day of incubation, the optical density (OD) of bacterial solutions measured at the wavelength of 600 nm using a spectrophotometer (7th-day data is nearly similar to 14th-day data therefore not mentioned in the graph)

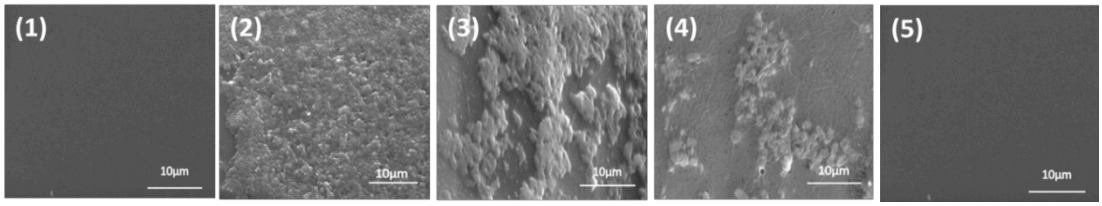


Figure 4b.5: Scanning electron microscopic images of biofilm developed on contact lenses incubated for 14 days: **(1)** Negative control : contact lens incubated in LB media (2 ml) and saline (2 ml) **(2)** Positive control: contact lens incubated in the presence of *P. aeruginosa* (OD_{600nm} : 0.1) in LB media (2 ml) and saline (2 ml) **(3)** CLCS control: contact lens incubated in the presence of *P. aeruginosa* (OD_{600nm} : 0.1) in LB media (2 ml) and CLCS (2 ml) **(4)** GC control: contact lens incubated in the presence of *P. aeruginosa* (OD_{600nm} : 0.1) in LB media (2 ml) and GC (0.05 mg/ml) in saline (2 ml) **(5)** contact lens incubated in the presence of *P. aeruginosa* (OD_{600nm} : 0.1) in LB media (2 ml) and GC (0.05 mg/ml) in CLCS.

4b.3.2. The anti-adhesion capacity of GC:

Biofilm inhibition study showed that GC may possess anti-adhesive property. Therefore, we performed anti-adhesion assay to confirm the activity. Coverslips prior incubated in GC solution showed reduced biofilm development on its surface indicating anti-adhesion potential of GC. Experiment data also revealed that in all the higher concentration sets after 2 mg/ml there was no any significant increase in biofilm reduction (Figure 4b.6). This may be because of saturation of GC on the coverslip, and any further higher concentration could not affect adhesion of bacteria to the surface.

GC concentration estimated through release study showed that though coverslip was incubated in higher concentration, after 2 mg/ml GC released from coverslip was constant, i.e. ~ 0.5 mg/ml (Figure 4b.6). This validated our result that there is no significant variation in biofilm reduction after 2 mg/ml due to saturation of GC.

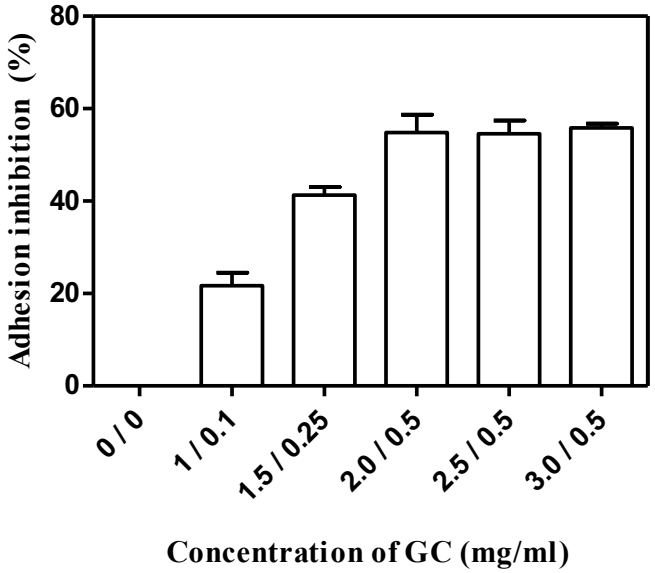


Figure 4b.6: Graph depicting anti-adhesion potential of GC (-/- on X-axis represents the concentration of GC used for prior incubation of coverslip/ concentration of GC estimated after release study)

4b.3.3. *In-vitro* cell proliferation and cytotoxicity of GC:

GC cytotoxicity was evaluated using *in-vitro* cell culture studies with mouse fibroblast cell line L929 and rat muscle cell line L6. Cell proliferation was evaluated by performing the MTT test. MTT data showed > 90 % survival of L6 cell line and > 80 % cell survival of L929 cell line till 200 mg/ml of GC (Figure 4b.7). This indicates greater biocompatibility of GC.

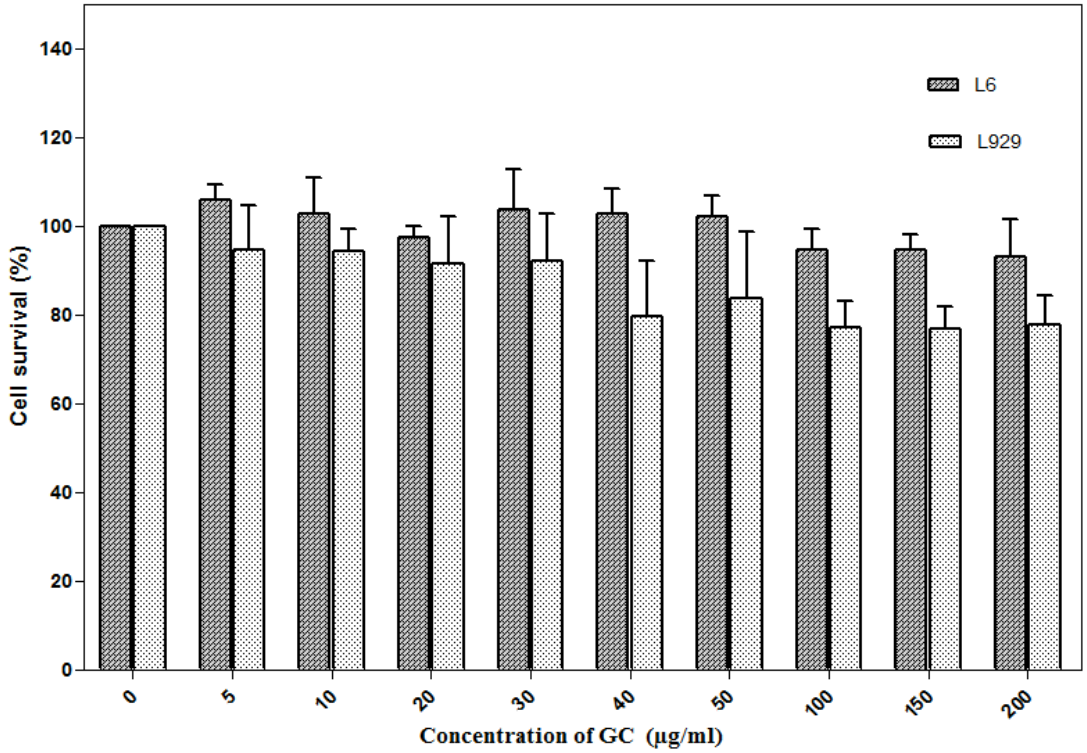


Figure 4b.7: Effect of increasing concentrations of GC (5, 10, 20, 30, 40, 50, 100, 150, 200 µg/ml final concentration) on cell survival (%) of mouse fibroblast cell line L929 and rat muscle cell line L6.

4b.4. Conclusion

Contact lenses have emerged as a promising solution to improve vision; however, it is frequently associated with severe corneal infections. Though, currently available contact lens cleaning solutions help to reduce CL associated eye infections, the occurrence of the infections not yet reduced completely. Therefore, some advancement in currently available cleaning solutions is extremely required. Current cleaning solutions usually contain antibiotics but as bacteria have developed resistance to most of the antibiotics, efficacy of cleaning solution has reduced.

Recently, quorum sensing inhibitors have emerged as a novel alternative to antibiotics but their application in contact lens cleaning solution to reduce biofilm have not yet tried. Therefore, in this chapter we have used novel QSI molecule- GC, along with contact lens cleaning solution to study its effect on biofilm development on contact lens. Combination of GC and contact lens cleaning solution reduces biofilm formation on contact lens till 14^{th} day which otherwise is not possible with

only contact lens cleaning solution. GC molecule not only retains its quorum sensing activity in contact lens cleaning solution but also inhibits biofilm formation by *P. aeruginosa*-a major etilogical agent in most of the eye infections. As GC has excellent biocompatibility, antiadhesive property, QSI and antibiofilm activity its supplementation in contact lens cleaning solution will improve efficacy of currently available contact lens cleaning solutions. Thus our work first time demonstrate QSI supplementation to contact lens cleaning solution to reduce biofilm associated eye infections.

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Thesis summary

AcSIR		Synopsis of Thesis
Name of the candidate		Ms. Amrita Patil
Enrollment number		10BB14A26047
Date of joining		25th July 2014
Thesis Title		Strategies for impeding quorum sensing in bacteria: Basic and applied aspects.
Research supervisor	Guide	Dr. Dhanasekaran Shanmugam
	Co-guide	Dr. Asmita Prabhune

Introduction

Earlier bacteria were thought to be present only in planktonic state i.e. as singular existence but research done in the last few decades has also revealed multicellular existence of bacteria. Bacteria possess a unique ability to communicate with each other through signal molecules called as quorum sensing (QS). This helps them to form multicellular structure surrounded by extracellular matrix, mainly consisting of polysaccharides and proteins which is called as biofilm[1]. QS is cell density dependent phenomenon in which at high cell density, concentration of signal molecules in the surrounding attains threshold level and it is detected by same or other bacterial species and that leads to autoinduction of quorum sensing system[1]. Bacteria produce different signal molecules and based on that there are four major quorum sensing systems. Auto inducer AI(I) present in Gram negative bacteria, AI (II) present in both Gram negative and Gram positive bacteria, AI (III) present in enteric gram negative and QS system present in Gram positive bacteria. In case of Gram negative bacteria, QS system mediated by AI (I) –Acyl homoserine lactone (AHL) signal molecules form the major class of quorum sensing system[2]. AHLs contain homoserine lactone group and fatty acid chains having various carbon chain length and functional group (oxo group) at 3' carbon of fatty acid. Each bacterium produce and detect specific signal molecules and trigger expression of different phenotypes such as swarming motility, secondary metabolite production, symbiosis, biofilm

formation and virulence factor production. Out of all mentioned phenotypes biofilm formation and virulence factor production plays major role in pathogenesis[2].

Statement of problem, Aims and Objective

Although antibiotics have become an integral part of modern healthcare, WHO's 2014 report on global surveillance of antimicrobial resistance stated that the world is entering into a post-antibiotic era in which infections which were curable with antibiotics for decades are again becoming incurable. Joint Programming Initiative on Antimicrobial resistance (JPIAMR) signed by 27 countries including India, is an example of global efforts aimed at reducing the emerging antimicrobial risks and preserving antibiotics for future generations[3]. Major reasons of antimicrobial resistance are

1. Selective pressure imposed by antibiotics: Antibiotics kill bacteria and therefore bacteria fight its best to evolve into a resistant strain.
2. Intra-Inter kingdom biofilm formed by pathogenic bacteria: Biofilm act as the shield around bacteria and even higher dose of antibiotics becomes ineffective

Therefore, approach of targeting quorum sensing has the advantage that it does not affect viability of bacteria but rather impede pathogenesis mediated through QS thus less likely to give selective pressure for resistance[4].

As quorum sensing mediate biofilm formation, compound having quorum sensing inhibitory activity can be used to avoid problem associated with biofilm. Although many reports on QS inhibitors have been published along with around 1000 patents, yet none of them has been commercialized[5,6]. Essential oil is one of the quorum sensing inhibitory molecules coming in the above mentioned category. Although essential oil possess quorum sensing inhibitory activity, medical preparations with only essential oil impose side effects therefore they are used with an emulsifier[7]. Choosing right emulsifying agent is again a challenge. Therefore methods which will modify essential oil itself to biocompatible form without affecting their biological activity are highly desirable for their use in medical preparations.

Major focus of my research was to develop highly efficient, stable as well as biocompatible quorum sensing inhibitory molecule which will have immediate applications in future. The main aim of my research was to convert major component of essential oil to hydrophilic form with increased bioavailability and to check

quorum sensing inhibitory activity of converted compound. Another objective was to find it's mode of action at molecular level so as to use it in the areas where biofilm associated problem is a major challenge. Out of many areas affected with biofilms I have chosen implants and contact lens surface.

Methodology used

1. Biosynthesis of novel glycomonoterpenes (G-citral and G-citron) was done by supplementing hydrophobic monoterpenes citral and citronellal to *Candida bombicola*
2. Conversion validation of monoterpenes, citral and citronellal to glycomonoterpenes G-citral and G-citron respectively was done by using TLC, oil displacement activity, HPLC, FTIR and structures of converted compound were predicted by doing NMR and MALDI-TOF
3. Quorum sensing inhibitory activity of G-citral and G-citron was explored by doing following assays

a) *Chromobacterium violaceum* (CV026) reporter strain based assay to check inhibition of quorum sensing mediated through C6HSL and C8 HSL signal molecules

b) *Agrobacterium tumefaciens* (pZLR4) reporter strain based assay to check inhibition of quorum sensing mediated through 3oxoC8 HSL, C10 HSL, and C12 HSL signal molecules

c) Pyoverdine inhibition assay

4. qRT PCR was used to decipher mode of action of G-citron on quorum sensing related gene expression
5. Novel G-citron and silk based coating was designed for biomedical implants
6. Synergistic effect of G-citron and lens cleaning solution to increase the antibiofilm effect

Key findings:

1. Biosynthesis of novel glycomonoterpenes using monoterpenes of essential oils and their characterization:

   Monoterpeness citral and citronellal (major components of lemongrasss essential oil) were converted to hydrophilic glycomonoterpenes G-citral and G-citron.

Characterization technique elucidated structure of newly synthesized glycomonoterpenes. G-citron contains three major lactonic forms with molecular weight 492.43, 473.47, and 330.39 Da whereas G-citral has an acidic form with molecular weight 389.33 and 346.23 Da

2. Exploring biological activity of glycomonoterpenes (G-citral and G-citron) as quorum sensing inhibitory molecules

   The glycomonoterpenes were able to individually inhibit QS, mediated through various medium-chains (C6 HSL, C8HSL) and long-chain (3oxoC8 HSL, C10HSL, C12 HSL) N-acyl homoserine lactones (AHLs). The glycomonoterpenes were also able to inhibit QS mediated phenotype i.e. biofilm formation and pyoverdine.

3. Deciphering mode of action of G-citron by studying its effect on quorum sensing related gene expression in *P. aeruginosa*.

   P. aeruginosa possess four quorum sensing pathway. qRT-PCR of cDNA synthesized from G-citron (0.1 mg/ml) treated *P. aeruginosa* showed significant inhibition of major quorum sensing associated genes lasI, lasR, rhlI, rhlR and also inhibited expression of biofilm associated genes rhlA, lecA, pelB. Thus, G-citron affected 4 quorum sensing pathway of *P. aeruginosa* viz. LasI/LasR, RhlI/RhlR, PQS, IQS. This data confirmed quorum sensing inhibitory activity of G-citron at molecular level.

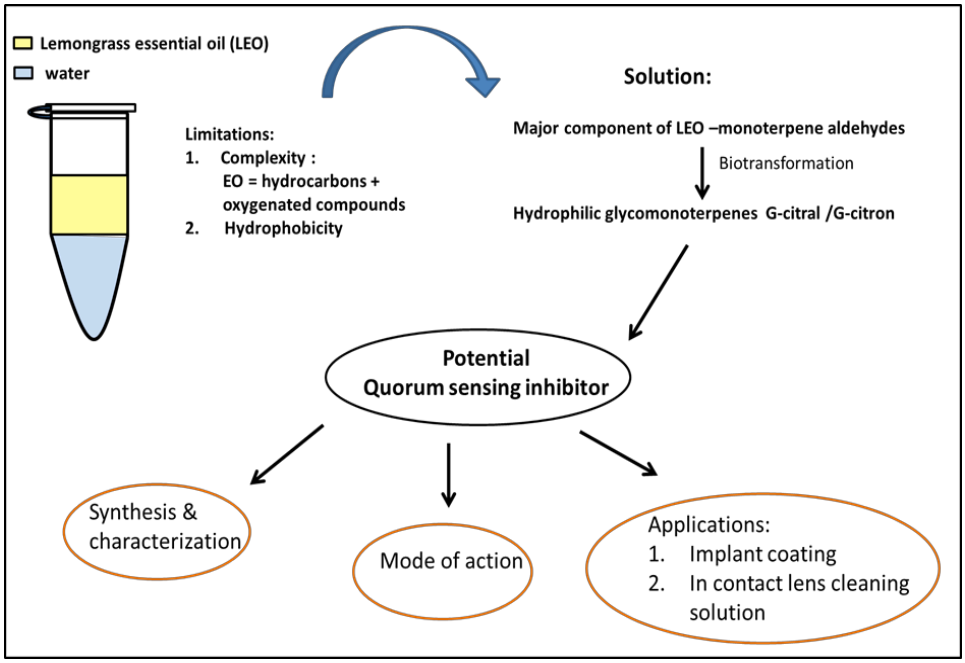
4. Development of novel G-citron based coating for biomedical implants:

   An innovative method of preparation of coating using two biological molecules viz. Silk fibroin and a glycolipid (GC)was developed. The coatings exhibited excellent anti quorum sensing activity and anti-biofilm activity. GC-SF coatings showed high mechanical strength, increase wettability, sustained release of GC and also showed proliferation of normal mouse fibroblast cells (L929) indicating its biocompatibilty. Thus our work demonstrates an innovative solution to simultaneously resolve multiple causes for failures of implant devices – biofilm formation, foreign body response and cracking of the coating.

5. Synergistic effect of G-citron and lens cleaning solution to increase the antibiofilm effect

   Soft contact lenses were incubated with only G-citron, only Contact lens cleaning solution and combination of contact lens cleaning solution (CLCS) and G-citron (0.05 mg/ml). Combination of CLCS and G-citron showed significant inhibition

of *P. aeruginosa* biofilm on contact lens. In this work in addition to QSI activity we have also demonstrated antiadhesive potential of GC which was observed by using scanning electron microscopy. The work presented here is the first report on use of QSI molecule (GC) in the contact lens cleaning solution.



References:

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Future prospects

Certain interesting leads encountered during the work and have significant potential have been described in detail

1. Anticancer potential of G-citron:

Citronellal's anticancer potential is reported earlier by Stone et al. (2013)[1]. Therefore, we compared anticancer potential of G-citron with its parent compound citronellal. For this study, we used human breast adenocarcinoma cell line (MCF7) (cancer cell line) and rat muscle cell line L6 (normal cell line) to find effective alternative, to conventional chemotherapy, that kills cancer cells without affecting normal human cells.

Concentration dependent effect of G-citron on normal and cancer cell line was investigated by MTT assay. It is the enzyme-based assay that determines mitochondrial dehydrogenase activity in the metabolically active living cells. In this assay, MTT, a yellow tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced into an insoluble dark purple color formazan product, by the action of NADPH dependent mitochondrial reductase enzyme. The formazan crystals are then solubilized using organic solvent (DMSO) and the absorbance is measured spectrophotometrically.

Cells with a density of $1x10^5$ cells/ml were seeded in complete DMEM media. and grown overnight on a 96-well plate. After 24 h incubation, cells were treated individually with increasing concentrations of G-citron and citronellal (5, 10, 20, 30, 40, 50, 100, 150, 200 µg/ml final concentration) at 37 °C with 5 % CO_2 for 24 h. Media was removed from all the wells and then 20 µl MTT (5 mg/ml) concentration was added to each well and incubated for 4 h at 37 °C. The cells were then treated with DMSO to solubilize the formazan crystals formed in viable cells and measured spectrophotometrically. Optical density was measured at 570 nm using SPECTRAmax plate reader (Molecular Devices Inc, USA) and cell survival percent and growth inhibition were calculated by using following formulae[2]. All experiments were conducted in triplicates.

$$\text{Cell survival (\%)} = 100 - \frac{(Control\ OD_{570\ nm} - Test\ OD_{570\ nm}) \times 100}{Control\ OD_{570\ nm}}$$

$$\text{Growth inhibition (\%)} = \frac{(Control\ OD_{570\ nm} - Test\ OD_{570\ nm}) \times 100}{Control\ OD_{570\ nm}}$$

In MTT assay, 50 µg/ml G-citron was sufficient to achieve 50 % survival inhibition of human breast adenocarcinoma cell line (MCF7) (Figure 1) however same concentration of G-citron did not affect survival of normal cell line rat muscle cell line L6 (Figure 2) as well as mouse fibroblast L929 (Figure 4b.7). MTT assay also showed that anticancer activity of G-citron was significantly higher than its parent compound. This indicates that conversion of hydrophobic citronellal to hydrophilic G-citron increases bioavailability of compound and therefore enhances anticancer activity. Further, to verify if the G-citron is more cytotoxic to tumor cells than to non-tumor cells, as expect for a good chemotherapy candidate, we evaluated the selectivity index (SI) between the two cell lines used -MCF7 (cancer cell line) and L6 (normal cell line) by using following formula[3]:

$$\text{Selectivity index} = \frac{LC_{50}\ \text{in normal cell line}}{LC_{50}\ \text{in cancer cell line}}$$

And as per calculations, G-citron showed high selectivity (SI = 8.6) than citronellal (SI= 2.08). A compounds with SI = >3.0 are considered as highly selective, whereas compounds with SI = <3.0 are considered non selective [1]. Thus, G-citron may become a good strategy in cancer therapeutic as compared to citronellal.

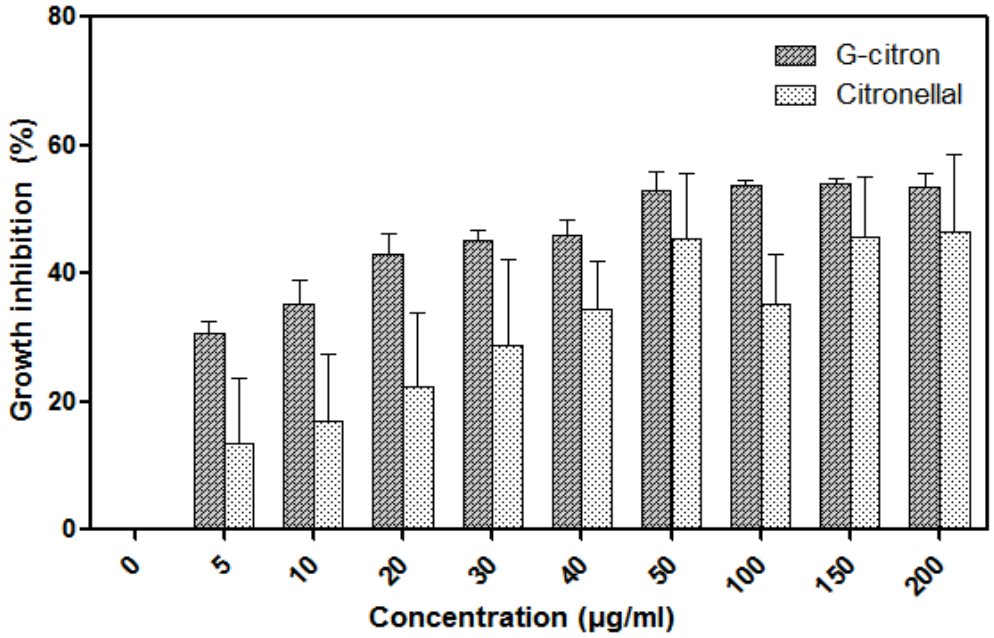

Figure 1: Result of MTT assay showing anticancer activity of G-citron and citronellal on human breast adenocarcinoma cell line (MCF7)

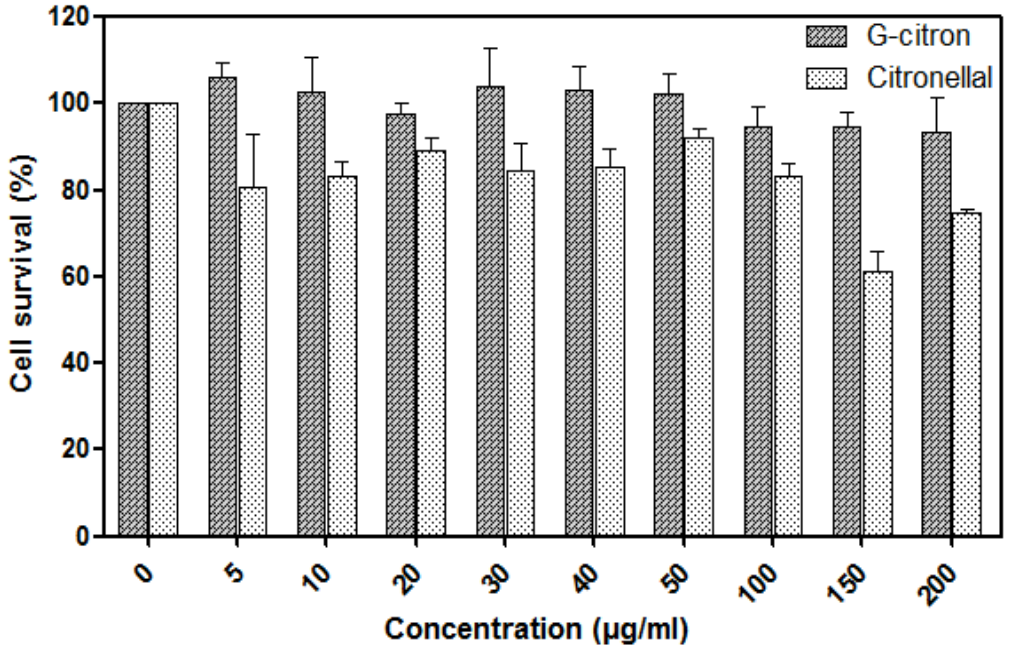

Figure 2: Result of MTT assay demonstrating cytotoxicity of G-citron and citronellal on rat muscle cell line L6

2. Inhibition of multispecies biofilm due to G-citron:

Escherichia coli, *Staphylococcus* spp and *Pseudomonas aeruginosa* (PA) are known to cause biofilm-related infections and they have been frequently isolated together from chronically infected wounds, cystic fibrosis and from indwelling medical devices[4,5]. Therefore in our study *E. coli, P. aeruginosa* and *S. epidermidis* were chosen for assessing multispecies biofilm inhibition by G-citron.

Initially, overnight grown cultures of *E. coli, P. aeruginosa*, *S. epidermidis* were added to LB medium in equal volume so as to achieve O.D. $_{600nm}$: 0.1. LB medium containing cultures (2 ml) was added to the plate where coverslip was placed. G-citron was added to the same plate and considered as test set however culture without G-citron was considered as control test. Both the sets were incubated for 24 h at 37 °C. After incubation, biofilms formed in control and treated plates were visualized by staining with crystal violet. The stained biofilm was then visualized under light microscope at ×400 magnification[6,7]. Multispecies biofilm inhibition was evaluated quantitatively by extracting crystal violet stain of biofilm by using 30 % acetic acid. O.D. was measured at 580 nm.

Results showed > 50 % biofilm inhibition due to G-citron (Figure 3) which indicate that G-citron also inhibits multispecies biofilm development.

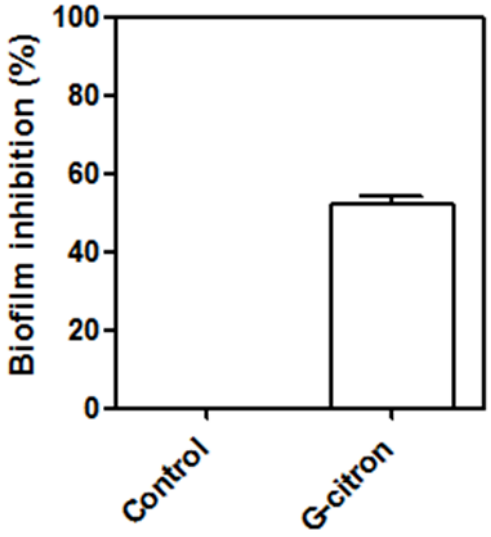

Figure 3: Quantitative evaluation of multispecies biofilm inhibition by G-citron.

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Publications

1. Mukherji R, **Patil A** & Prabhune A. Role of extracellular proteases in biofilm disruption of Gram positive bacteria with special emphasis on *Staphylococcus aureus* biofilms. ***Enzyme Engineering*** 4, 1-7 (2015) doi:10.4172/2329-6674.1000126
2. Maria Borsanyiova, **Amrita Patil**, Ruchira Mukherji, Asmita Prabhune, Shubhada Bopegamage Biological activity of sophorolipids and their possible use as antiviral agents ***Folia Microbiologica*** 1-5 (2015) doi: 10.1007/s12223-015-0413
3. Niharika Singh, **Amrita Patil**, Asmita Prabhune, Gunjan Goel Inhibition of quorum sensing mediated biofilm formation in *Cronobacter sakazakii* strains ***Microbiology*** 162, 1708-1714 (2016) doi: 10.1099/mic.0.000342
4. Niharika Singh, **Amrita Patil**, Asmita Prabhune, Gunjan Goel Diverse profiles of N-acyl-homoserine lactones in biofilm forming isolates of *Cronobacter sakazakii* ***Virulence*** 1-7 (2016) doi: 10.1080/21505594.2016.1226713
5. **Amrita Patil,** Kasturi Joshi, Ruchira Mukherji, Asmita Prabhune Biosynthesis of glycomonoterpenes to attenuate quorum sensing associated virulence in bacteria. ***Applied Biochemistry and Biotechnology*** 181, 1533-1548 (2016) doi: 10.1007/s12010-016-2300-8
6. Emmanuel Joseph, **Amrita Patil**, Swarali Hirlekar, Abhijit Shete, Nimisha Parekh, Asmita Prabhune, Anuya Nisal Glycomonoterpene-functionalized crack-resistant biocompatible silk fibroin coatings for biomedical implants ***ACS Applied Bio Materials*** 2, 675-684 (2019) doi: 10.1021/acsabm.8b00515 (E.J. and A.P. contributed equally)

Patent:

Provisional application filed: Bioactive glycoderivatives of terpenes and terpene alcohols and method of synthesis thereof. Application number 3894/DEL/2015

Poster presentations:

Amrita Patil, Ruchira Mukherji, Asmita Prabhune, Best poster award for the poster titled 'Incapacitating pathogenic bacteria using glycomonoterpenes: Are we tackling MDR??' on Science day (2016) poster presentation at CSIR-National Chemical Laboratory, Pune-India