9 SUMMARY AND CONCLUSION
The magic wand of antibiotics, discovered back in 1930s, thrilled the human race with its remarkable power of treating bacterial infections that were causing enormous deaths in the 19th century. However, the outbreak of antibiotic resistance among bacteria, soon in 1990s, conquered the magic power of these drugs, and further upsurge in the resistance phenomenon led to the emergence of multidrug resistance, now extended by the extreme and total drug resistance. The present dilemma of evolving drug resistance pose a serious issue, and urgent measures need to be undertaken for combating this problem. Preventive measures such as maintaining proper hygiene, avoiding antibiotics in normal viral flu, or mild respiratory problems, completing the prescribed antibiotic course and educating people about the problem and the importance of these measures, have been recommended by the U.S. FDA, to control further aggravation of the problem. Apart from these, the development of new drugs is being demanded to effectively fight the resistant bacterial infections (Anonymous 2011).

Targeting the organism’s virulence is being recognized as an alternative infection therapy, wherein bacterial growth is not affected, but its ability to produce toxins and disseminate the infection is hampered. This helps to control the infection and its harmful effects, and in the meanwhile, the host immune system can play its role in clearing out the pathogen from the body. The quorum sensing (QS) pathway of gene regulation in bacteria has been recognized as an important anti-virulence target, due to its key role in the expression of virulence and biofilm development in many pathogenic bacteria (Cegelski et al. 2008).

The present study has been focussed on the disruption of QS in MDR, nosocomial pathogens, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, using plant products. These pathogens are aerobic, Gram negative bacteria that generally cause opportunistic infections, affecting the immunocompromised individuals. They have been commonly implicated in the hospital acquired infections, and are often associated with resistance to multiple drugs, creating problems in their treatment and prolonging the stay and suffering of the hospitalized patients.
*P. aeruginosa* and *A. baumannii* produce the QS signalling molecules called acyl homoserine lactones (AHLs or acyl-HSLs). At the threshold level, AHLs bind and activate transcriptional regulators, which are directly responsible for the co-ordinated expression of genes involved in the virulence and biofilm maturation of the organism (van Delden & Iglewski 1998; Niu et al. 2008). Thus, interfering with the AHL production or AHL binding with the transcriptional regulators will affect the production of virulence factors or biofilm maturity of the organisms. Realizing the potential of QS inhibitors as anti-virulence drugs, many studies have been carried out to discover natural and synthetic inhibitors of QS regulated virulence and biofilm in many Gram negative bacteria (Lasarre & Federle 2013). Among all of these, many have been effective in treating infected animal models, while as per the published data available, only azithromycin has been shown to possess clinical efficacy in preventing pseudomonal pneumonia (van Delden et al. 2012). Thus, there is demand for more anti-QS agents that can acts as potential drug candidates and the present study was undertaken as the basic research in the discovery of novel, plant based QS inhibitor/s.

In the present study, five Indian medicinal plants, Manjistha, Guduchi, Katuka, Kanchanara, and Aragvadha, were assessed for their anti-QS efficacy w.r.t. the pathogens under study. Since many Gram negative bacteria that produce AHLs, follow the similar LuxR/I mechanism of QS, it was hypothesized that the inhibition of short acyl-HSLs in the biomonitor strain, *Chromobacterium violaceum*, would correspond to the general inhibition of the short acyl-HSLs pathway. For long acyl-HSLs, *P. aeruginosa* and *A. baumannii* were exposed to the plant extracts and the AHL produce was detected by the reporter strain, *Escherichia coli* MG4/pKDT17. Different solvents and extraction procedures facilitated the extraction of phytochemicals on the basis of polarity and heat stability; as a result, varying bioactivities were observed for each of the extract. Garlic extract, which was used as the positive control for QS inhibition, provided a cut-off for selecting the plant extracts for further work of the project. Among all the plant extracts, the ethyl acetate extracts of Guduchi exhibited higher potential than garlic extract in inhibiting both the short acyl-HSLs and long acyl-HSLs. Further, the cold ethyl acetate extract of Guduchi (GCEE) did not exhibit any bactericidal or bacteriostatic effect, which
confirmed the anti-QS potential of the plant extract. So, GCEE was taken forward in the further objectives of the study.

Since it is known that the AHL-QS regulates the production of extracellular virulence factors in *P. aeruginosa* and biofilm formation in both, *P. aeruginosa* and *A. baumannii*, the study was further directed towards evaluating the effect of QS inhibitory extract, GCEE, on the bacterial virulence and biofilm development. Along with the reference strain, the clinical strains of the above pathogens were also included as the test organisms. While GCEE was effective in inhibition of *P. aeruginosa* virulence in all the test strains, it was found to stimulate biofilm formation in the organism, which may be attributed to the carbohydrates detected in the plant extract, along with the varying bioactivities of other phytochemicals of the extract. Although, GCEE was able to inhibit biofilm formation in the reference strain of *A. baumannii*, it exhibited varying effects on the clinical strains. The plausible explanation for the results could not be provided due to the insufficient molecular information available w.r.t. *A. baumannii* biofilms. Nevertheless, it could be concluded that the QS inhibitory potential of GCEE led to the further reduction in bacterial virulence of *P. aeruginosa* and biofilm formation of *A. baumannii*; and it may be hypothesized that the separation of the interfering compounds in the plant extract would assist in the biofilm inhibition of *P. aeruginosa*. A static mode of biofilm formation, using microtiter plate, was used in the present study. A continuous flow system can be employed to study the efficacy of plant extract on bacterial biofilms under the conditions of continuous flux of nutrients, plant extract and the removal of exoproducts.

The phytochemical profiling of GCEE was performed using chromatography techniques, revealing the presence of carbohydrates and terpenoids in the extract. The plant, Guduchi, is known to contain many active principles belonging to the terpenoid classes such as terpenoid glycosides, diterpenoid lactones, sesquiterpenoids, etc (Grover & Bansal 2012). So, it was concluded that the terpenoids might be responsible for the anti-QS activity of the extract. Further, the HPTLC fingerprinting of the extract provided a valuable reference for future studies on the extract.
The final goal of the project was to fractionate and identify the anti-QS phytochemical/s in the plant extract, which was achieved by bioassay guided fractionation of the extract, followed by GC-MS detection of the compounds in the most active fraction. Literature reviewing and molecular docking provided significant assistance in predicting the compounds likely to play an important role in the QS inhibitory effects of the plant. The active molecules were found to be concentrated in one of the fractions obtained using column chromatography, since a single fraction exhibited all the three bioactivities, anti-QS, anti-virulence and anti-biofilm, while unaffected bacterial growth. Fractionation of GCEE facilitated the separation of the molecules interfering with the anti-biofilm activity of the QS inhibitors. As a result, the active fraction was able to inhibit \textit{P. aeruginosa} biofilm formation. GC-MS analysis of the active fraction revealed the presence of aliphatic compounds, such as methyl esters of fatty acids, carboxylic acid derivatives, and some phenolic compounds. While fatty acid esters have been previously detected in the plant, the other compounds were detected for the first time. However, these compounds need to be isolated in their pure form and their identity confirmed by NMR spectroscopy, before claiming the presence of these compounds in the plant. Thus, the isolation and validation of the constituents in the active fraction presents a significant future scope for the study.

A reviewing of the literature available on the bioactivities of the compounds detected in the active fraction, hinted towards the probable role of fatty acids in QS inhibition. Previously, some unsaturated and branched chain fatty acids have been reported to possess anti-QS and anti-biofilm potential at micromolar or even nanomolar concentrations (Davies & Marques 2009; Inoue et al. 2012; Singh et al. 2013). Thus, it was speculated that the fatty acids detected in the present study, might play a significant role. However, when pure standards of hexadecanoic acid and octadecanoic acid were tested for long acyl-HSL inhibition, they failed to show significant effect on the AHL production in the bacteria under study. So, for deducing the probable QS inhibitors, docking studies were performed, wherein based on the interactions of the compounds with the QS proteins (LasR, LasI), docking scores were obtained. High docking scores and the interactions such as hydrogen bonding, with the ligand binding sites of the
proteins, were exhibited by some of the compounds, suggesting their high binding affinity for the QS proteins. Thus, it was concluded that the compounds, viz. methyl 16-methylheptadecanoate, methylhexadecanoate, 2-(5-ethenyl-5-methyloxolan-2-yl) propan-2-ol and 2,3,4-triacetoxybutyl acetate were probably responsible for the anti-QS activity exhibited by the extract.

A lot of medicinal research has been directed towards the plant metabolites, due to their complex nature and the notable bioefficacies, which have rendered the enormous medicinal applicability of various plants since ancient ages. The anti-QS screening of five medicinal plants in the present study unveiled a new bioefficacy for the well-known immunomodulatory plant, *Tinospora cordifolia* (common name – Guduchi), with further potential to restrain QS regulated virulence and biofilm development in the pathogens. Recently, in a study by Maeda et al. 2012, mutations in the *mexR* and *nalC* genes in the marker strain, *P. aeruginosa* PAO1, led to a high efflux of the well characterized QS inhibitor, C30 furanone. The *mexR* and *nalC* act as negative regulators of the MexAB-OprM multidrug efflux pump. Resistance to C30 and 5-fluorouracil was also reported in the clinical strains of *P. aeruginosa*, suggesting that the phenomenon of resistance to QS inhibitors exists in bacteria, even before the exposure to the inhibitors (García-Contreras et al. 2013). More extensive studies are required to evaluate the effects of QS inhibitors on larger number of clinical strains, and more insights into the different mechanisms of resistance in the clinical strains will also be helpful to develop further drug candidates.

The compounds suggested as the probable QS inhibitors by docking investigation in the present study, may be chemically synthesized and further evaluated using biological experimentation, to confirm their role in QS disruption. Studies on the individual and synergistic effects of the compounds would be valuable in developing effective drug candidates.

Gene expression studies may be performed to depict the mechanisms for the inhibitory actions of the compounds. Real time PCR and microarray studies would help in
understanding the effects of these compounds on the expression of the core QS genes (*luxI* and *luxR* homologues) as well as the genes regulated by the QS pathway.

*P. aeruginosa* has been found to contain a third QS system, which produces a quinolone signal, and has been related with the regulation of virulence genes, as well as the AHL systems (Strateva & Mitov 2011). Hence, further work may focus on the effects of the AHL inhibitors on the quinolone signalling of the organism. It has been shown that the las system controls the regulation of the quinolone system; hence, inhibitors of the las system will directly affect the quinolone system. However, studies based on the effect of compounds on the quinolone signalling, independent of the AHL inhibition, is also recommended.

Gram negative bacteria such as *Yersinia pestis, Serratia marcescens, Burkholderia cepacia*, etc. are known to produce AHL-QS systems that regulate the virulence phenotypes in these bacteria. Hence, it may be hypothesized that the AHL inhibitors of *P. aeruginosa* and *A. baumannii* would be effective in controlling virulence of these bacteria, thus broadening the spectrum of their activity. So, experimental studies pertinent to this prospect may be carried out.

The compounds that prove to be effective as QS inhibitors in *in vitro* assays using bacteria can be forwarded to further screening on cell line models wherein cell viability of infected epithelial cells, in the presence or absence of QS inhibitors, can be evaluated. Co-culture cell line model such as macrophages cultivated with the alveolar epithelial cells has been developed to study the effect of AHLs on the host innate immunity (Crabbé et al. 2011). If such co-cultured models are developed to study infections, it will not only help to reduce animal use, but prove to be more relevant to the *in vivo* situation. The compounds screened from the *in vitro* cell line assays can be subjected further to *in vivo* studies using different infection models, such as nematodes, insects, rodents, and subsequently developed into potential drug entities for the treatment of bacterial infections.