Summary and Conclusion
SUMMARY

Till early nineties, it was believed that plant as a sessile and single biological entity but improved scientific knowledge and burgeoning research interest envisioned plant do harbour untold number of microorganisms which dwell on their surface, across their roots and inner tissues. Since then plant and microbe interaction has fostered the researcher towards harnessing the valuable potentials bearing pharmaceutical importance. One such area gaining impute importance is endophytic plethora which remains largely untapped reservoir in recent years research on these inhabitants has begun to appreciate their roles. Fuelling progress in endophytes has envisioned them as one of the highly merited microbial habitants. One of the prime aspects of endophytes is their role in secretion of value added secondary metabolites bearing biological activities. In spite of their progress in biological sciences yet one of the least studied areas in the field of endophyte is their evaluation towards synthesis of nanoparticles. Interaction of endophytes and nanoscience is new area which can open a new avenue.

Hence the present study aims to evaluate bacterial endophytes from five medicinal plants of India region. The findings of the present investigation are promising and add tremendous importance towards growing scientific knowledge on endophytes and their emerging role in synthesis of silver and gold nanoparticles. With scanty reports available on the endophytes for synthesis of nanoparticles, an attempt made in the present study by evaluating endophytic bacteria for synthesis of nanoparticles and their antimicrobial activity against important selected test pathogens was successfully achieved to report biogenic based principle for the synthesis of nanoparticles as an alternative approach for popular conventional methods which bound with various limitations. The present study reports 3 potent endophytic bacteria Pseudomonas veronii strain AS 41G, Pseudomonas fluorescens strain CA 417 and Aneurinibacillus migulanus MB 141 capable of rapid synthesis of nanoparticles within 30 minutes of incubation time.

Among the isolates reported two isolates Pseudomonas veronii strain AS 41G and Aneurinibacillus migulanus MB 141 are novel and the present study forms the first report on these bacteria as an endophytes globally and forms the first report of
their evaluation for synthesis of nanoparticles. Along with nanoparticles synthesis in the present investigation prime emphasis was attributed for isolation of antimicrobial metabolites from endophytic bacteria via bio-assay guided fraction techniques coupled with its characterization using hyphenated techniques which resulted in secretion of antimicrobial metabolite from *Pseudomonas veronii* strain AS 41G which was surveyed and matched to reveal its close affinity to benzoquinone derivative DDQ which forms the first report from this bacterium capable of secreting this antimicrobial metabolite. Similarly antimicrobial metabolite was secreted by *Pseudomonas fluorescens* strain CA 417 which was surveyed and matched to reveal its close affinity to polyketide 2,4-DAPG. These two metabolites showed significant antibacterial and antifungal activity against test pathogens. With this brief summary the conclusion of present investigation is designed and reported based on chapters as follows

**CONCLUSION**

**Chapter one**

The present chapter summarizes the general introduction of the doctoral study which comprises the basic notion of the reason for selecting the present theme followed by the role of bioprospecting microbial diversity and highlighting the emerging importance of plant associated microorganisms and highlighting the significance of endophytes and their activity, role of bacterial endophytes capable of secreting antimicrobial metabolites and their characterization. Envisioning less exploited area of these endophytes such as synthesizing nanoparticles. Brief introduction of nanotechnology and its intersection with interdisciplinary fields of sciences and its important aspect pertaining to nanoparticles followed by protocols for nanoparticle synthesis and their associated limitations with employed conventional methods and burgeoning interest on biological benign process for synthesis of nanoparticles and evaluation of nanoparticles for antimicrobial activity which has been one of the major area of concern in the current scenario with the emergence of drug resistant microorganisms. The outcome of the chapter is reported in the form of two review articles.
Chapter two

The second chapter comprises of selected medicinal plants viz., *Annona squamosa* L., *Coffee arabica* L., *Tridax procumbens* L., *Euphorbia hirta* L., *Mimosa pudica* L. and their reasoning for selecting in the present investigation. The selected plant materials were subjected to surface sterilization and observed for emergence of bacterial endophytes on nutrient media supplemented with cycloheximide and Bavistin which suppressed the growth of fungal endophytes. In total three hundred and thirty two bacterial endophytes were screened and isolated and designated with alpha numeric code based on their source of isolation. All the endophytes obtained were screened for the synthesis of silver and gold nanoparticles which was achieved by growing the isolates onto the media supplemented with metal salts such as silver nitrate and gold chloroaurate. The colonies capable of growing onto these enriched media were further selected for large scale fermentation and the supernatant was evaluated for synthesis of silver and gold nanoparticles. The results revealed among the 332 isolates, 41 endophytes were capable of growing onto the enriched media in primary screening.

Whereas only 7 endophytes were further capable of synthesizing the nanoparticles at secondary screening. Based on the optimization studies 3 endophytes were selected for further experiments and characterization of these bioactive isolates was carried out by using set of universal primers as described in materials and method. The obtained 16S rRNA gene sequence was matched at NCBI using BLAST tool to reveal its maximum similarity. Acquisition of AS 41G sequences was 99% homologous to gene sequence of *Pseudomonas veronii* strain CIP 104663.

The strain CA 417 showed 99% homologous to 16S rRNA sequence of *Pseudomonas fluorescens* strain BCPBMS. Whereas isolate MB 141 showed 99% homologous to the *Aneurinibacillus migulanus* strain 2012 BaDB 21. All the homologous was carried out with the database available online at NCBI site. Based on the results acquired from molecular characterization, each isolate was designated with generic and species nomination, the sequences of all three strains was deposited at Genbank and received accession numbers for *Pseudomonas veronii* strain AS 41G with KC 480604, *Pseudomonas fluorescens* strain CA 417 with KC 480603 and
Aneurinibacillus migulanus strain MB 141 with KF 606762. These endophytes were capable of reducing the metal salts and synthesize nanoparticles within 30 minutes of incubation time under optimized parameters.

Primary screening of antimicrobial activity from isolated endophytes resulted in 12 endophytes exhibiting activity with agar over lay assay against test bacteria and dual culture technique for test fungus. But secondary screening resulted in 4 endophytic bacteria exhibiting antimicrobial activity among these four two endophytic bacteria were capable of showing activity against only Gram-positive bacteria whereas rest of the two endophytes showed activity against all the test pathogens. Interestingly the selected isolates were also capable of synthesizing nanoparticles as mentioned above Pseudomonas veronii strain AS 41G and Pseudomonas fluorescens strain CA 417 which becomes subject of interest for further fermentation, isolation and characterization of secondary metabolites bearing antimicrobial activity.

Chapter three

The selected endophytic isolates Pseudomonas veronii strain AS 41G, Pseudomonas fluorescens strain CA 417 and Aneurinibacillus migulanus strain MB 141 were cultured and cell free supernatant was treated with 1 mM of silver nitrate and gold chloroaurate respectively and incubated under optimized parameters. Samples were drawn periodically to observe the color change and confirmed with UV-Visible spectrophotometer to observed absorbance between 200 to 700 nm for silver nanoparticles and 300 to 800 nm for gold nanoparticles resulting in ascertained peak between 350 to 550 nm for silver nanoparticles and 450 to 550 nm for gold nanoparticles with Pseudomonas veronii strain AS 41G. Similarly Pseudomonas fluorescens strain CA 417 showed peak in the range of 150 to 300 nm for silver nanoparticles and 450 to 550 nm for gold nanoparticles.

Whereas Aneurinibacillus migulanus strain MB 141 showed absorption peak in the range of 350 to 550 nm for silver nanoparticles and 450 to 600 nm for gold nanoparticles. Interestingly the present findings forms first report on Pseudomonas veronii strain AS41G and Aneurinibacillus migulanus strain 141 as an endophyte and their evaluation for synthesis of nanoparticles. The synthesized nanoparticles were
characterized using hyphenated techniques resulting in polydispersed nanoparticles with size ranging from 5 to 50 nm, 10 to 50 nm for silver and gold nanoparticles respectively. FTIR analysis predicted the role of biomolecules in synthesis and stabilization of nanoparticles and XRD results showed crystalline nature of nanoparticles. Both silver and gold nanoparticles were evaluated for antibacterial and antifungal activity which resulted in moderate antibacterial activity with both silver and gold nanoparticles. But no antifungal activity was conferred with both silver and gold nanoparticles.

Among the test organisms evaluated, *Klebsiella pneumoniae* was more sensitive to silver nanoparticles which showed more activity than standard gentamicin. Whereas gold nanoparticles exhibited significant activity against *Pseudomonas aeruginosa* compared to gentamicin. Minimal inhibitory concentration resulted in lowest concentration of the silver and gold nanoparticles to inhibit the test pathogens which varied from 31.25 µg/ml to 250 µg/ml. Nanoparticles mode of action was evaluated by treating the 10ng DNA of *Staphylococcus aureus* (MTCC 7443) with 10 µl of silver and gold nanoparticles and analyzed using 1% agarose gel electrophoresis which showed deformed and damage of DNA with light colored band compared to control DNA which indicated the action of nanoparticles on DNA.

**Chapter four**

Antimicrobial metabolites were extracted from fermentation broth of *Pseudomonas veronii* strain AS 41G and *Pseudomonas fluorescens* strain CA 417 using liquid-liquid extraction protocol with ethyl acetate as a solvent to obtain organic phase which was reduce and the ethyl acetate extract was subjected to bio-assay guided fractionation using column chromatography, TLC, HPLC and bioautography techniques resulted in purification of bioactive fraction bearing activity. Characterization of bioactive fraction with hyphenated techniques resulted in chemical profiling of bioactive fractions.

*Pseudomonas veronii* strain AS 41G secreted antimicrobial metabolite which was subjected to column chromatography as yellow color fraction within the range of 12.5 to 16 % of ethyl acetate in hexane. Further HPLC analysis revealed the elution time of the bioactive fraction between retention time ($t_R$) 6- 8 minutes. LC-MS
chromatogram revealed intense peak with molecular mass of m/z 227. Since it is a non-protonated secondary metabolite hence does not contain any proton moiety which resulted in no protons signals were detected further $^{13}$C NMR (DMSO-$d_6$): 134.00 (C-1), 137.19 (C-1’), 118.36 (C-2), 117.76 (C-2’), 180.47 (C-3), 172.43 (C-4). Based on the results ascertained with the characterization techniques mentioned above, whose findings was surveyed and matched with the metabolite in chemical library to reveal its close affinity to benzoquinone derivative as 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) with molecular formula C$_8$Cl$_2$N$_2$O$_2$. The characterized DDQ showed activity against all the test pathogens with significant activity was conferred against *Staphylococcus epidermidis* (MTCC 435) compared to standard gentamicin. Bioautography assay revealed the bioactive fraction at $R_f$ 0.5 with activity against *Staphylococcus aureus* (MTCC 7443). To best of our knowledge the present study forms a first report on *Pseudomonas veronii* strain AS 41G secreting DDQ bearing antibacterial and antifungal activity.

*Pseudomonas fluorescens* strain CA 417 secreted antimicrobial metabolite which was subjected to column chromatography to elute bioactive fraction within the range of 60 to 65% of ethyl acetate in hexane. Further HPLC analysis revealed the elution time of the bioactive fraction between retention time ($t_R$) 4-5 minutes. LC-MS chromatogram revealed an intense peak with m/z=211.13 [M+H] in positive mode. $^1$H-NMR spectrum showed four singlet at 6H for acetyl moiety and aromatic protons of benzene ring $^{13}$C-NMR showed six carbon atoms of benzene ring and its associated acetyl groups. Based on the results ascertained with the characterization techniques mentioned above, whose findings was surveyed and matched with the metabolites in chemical library to reveal its close affinity to the metabolite as 2,4 diacetylphloroglucinol (2,4-DAPG) with molecular formula C$_{10}$H$_{10}$O$_5$. The characterized 2,4-DAPG showed activity against all the test pathogens with significant activity was conferred against *Shigella flexneri* (MTCC 1457) compared to standard gentamicin. Bioautography assay revealed the bioactive fraction at $R_f$ 0.8 with activity against *Staphylococcus aureus* (MTCC 7443).
SIGNIFICANCE OF THE PRESENT INVESTIGATION

The outcome of the present study is promising enough to report two isolates *Pseudomonas veronii* strain AS 41G and *Aneurinibacillus migulanus* MB 141 are novel and the present study forms the first report on these bacteria as endophytes globally and their evaluation for synthesis of nanoparticles. The synthesis protocol forms ecofriendly and biogenic based principle for synthesis of nanoparticles. The reduction of metal salts was rapid compared to majority of reports pertaining to microbial mediated synthesis of nanoparticles. To best of our knowledge the present findings forms the first report on bactericidal activity of nanoparticles on phytopathogenic bacteria. Similarly the present study forms the first report on DNA damage activity of biologically synthesized nanoparticles on DNA of *Staphylococcus aureus* (MTCC 7443). Along with nanoparticles synthesis in the present investigation prime emphasis was attributed for isolation of antimicrobial metabolites from endophytic bacteria via bio-assay guided fractionation coupled with its biophysical characterization using hyphenated techniques which resulted in secretion of antimicrobial metabolite from *Pseudomonas veronii* strain AS 41G which was surveyed and matched to reveal its close affinity to benzoquinone derivative DDQ which forms the first report from this bacterium capable of secreting this antimicrobial metabolite. *Pseudomonas fluorescens* strain CA 417 secreted antimicrobial metabolite which was surveyed and matched to reveal its close affinity to polyketide 2,4-DAPG. Both the antimicrobial metabolites showed significant activity against phytopathogens compared to tetracycline. The results obtained in the present investigation has been communicated in the form of research articles with few are online and some of the articles are under pipeline of publishing and submitted to journals.