SUMMARY AND CONCLUSIONS

The present study was aimed at isolation and identification of antimicrobial metabolites from endophytic fungi from plant sources. The studies focus on variation in diversity and distribution of endophytic fungi in different seasons such as summer, monsoon and winter. Endophytic fungi were isolated from stem and leaves of *Mirabilis jalapa* and *Ficus pumila* and screened for antimicrobial activity. The primary screening was carried out using dual agar culture technique. Bioactive endophytic fungi were fermented on potato dextrose broth medium to obtained ethyl acetate extracts of culture broth. In secondary screening, ethyl acetate extracts were evaluated for their antimicrobial activity against a panel of human and phytopathogenic fungi and bacteria by disc diffusion method.

Three species of *Xylaria* FPL-10(S), FPL-25(M), FPL-52(S) and *Phomopsis* sp., FPS-25(M) were selected as potent antimicrobial endophytes for isolation and characterization of antimicrobial active principle and PCR based molecular characterization of the species identification of the endophytes.

A functional gene-based molecular screening strategy was used to target type I polyketide synthase (PKS) genes in bioactive fungal endophytes. Bioinformatic analyses of these biosynthetic pathways facilitated inference of the potential bioactivity of endophyte natural products, suggesting that the isolated endophytes are capable of producing a plethora of secondary metabolites. In addition, antimicrobial potentiality of the compound isolated was also evaluated by determining the MIC of the compound in comparison with standard drugs against a panel of test organisms.

First chapter deals with the general introduction on role of natural products and its sources. Similarly role of microbial natural product in drug discovery from diverse habitats including plant associated microbes such as endophytes. Significance of endophytic fungal bioactive secondary metabolites in drug discovery was also mentioned.

Second chapter deals with the isolation and identification of endophytic mycoflora associated with *Mirabilis jalapa* Linn. and *Ficus pumila* Linn. Distribution and diversity of endophytic mycoflora assessed and quantified using diversity indices viz., Simpson’s Dominance index, Shannon-weiner index and species richness. A total of
two hundred thirty eight (238) (19.83%) endophytic isolates were collected from 1,200 plant tissue samples of stem (600 segments) and leaves of (600 segments) *Mirabilis jalapa* Linn. (Nyctaginaceae) and *Ficus pumila* Linn. (Moraceae) in three different seasons namely summer, monsoon and winter from Mysore, Karnataka, southern India. 238 endophytic isolates were categorised into 23 taxa, comprising 3 ascomycetes genera (species of *Chaetomium*, *Sporormia* and *Xylaria*) (10.92%), 5 coelomycetes genera (species of *Colletotrichum*, *Pestalotiopsis*, *Phoma*, *Phomopsis* and *Phylosticta*) (18.06%), 11 hyphomycetes genera (species of *Acremonium*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Curvularia*, *Drechslera*, *Fusarium*, *Myrothecium*, *Nigrospora*, *Penicillium* and *Trichoderma*) (55.46%), 2 zygomycetes genera (species of *Mucor* and *Rhizopus*) (2.94%) and 2 morphospecies of *Mycelia sterilia* (8.4%).

*Aspergillus flavus* and *Xylaria* spp. were dominant in leaf tissue, whereas species of *Fusarium* and *Pestalotiopsis* were dominant in stem tissues of *Ficus pumila* and *Mirabilis jalapa* in terms of overall colonization frequency. Seasonal distribution studies revealed that foliar endophytic mycoflora was higher in terms of isolation and colonization rates. Simpson index and Shannon-wiener index exhibited significant difference in stem and leaf tissue of *Ficus pumila*. The species richness was high in stem and leaf tissue of *Mirabilis jalapa* representing 19 different endophytic fungal taxa. Taxonomic composition survey revealed that endophytic fungi belonging to the class hyphomycetes dominated in stem and leaf tissues. The recovery of endophytes was greater in leaf tissue than in stem.

In the third chapter, endophytic fungal isolates were subjected to primary screening for antimicrobial activity using dual agar culture assay. In the preliminary screening, twenty five out of two hundred thirty eight endophytic isolates exhibited antimicrobial activity against one or the other test pathogen. Twenty five endophytic isolates selected by primary screening were subjected to fermentation on potato dextrose broth. Ethyl acetate extracts of culture broths of endophytic fungi were evaluated for antimicrobial activity in secondary screening against a panel of human and phytopathogenic bacteria and fungi using disc diffusion assay. Four endophytic fungal isolates three species of *Xylaria* and (16%) viz., FPL-10(S), FPL-25(M), FPL-52(S) and
one species of *Phomopsis* FPS-25(M) out of 25 were exhibited broad spectrum antimicrobial activity against the test microbes.

**Fourth chapter** deals with molecular characterization and amplification of ketosynthase domain sequence from bioactive endophytic fungal polyketide synthase gene. The reliability of morphological identification of three different genera *Pestalotiopsis*, *Phomopsis* and *Xylaria* were confirmed by molecular characterization by amplification of rDNA-ITS fragment using PCR and phylogenetic analysis. The accession numbers obtained were *Pestalotiopsis* sp. FPS-88(M) JQ723317, *Xylaria* sp. FPL-10(S) JX839538 and *Xylaria* sp. FPL-25(M) KF564637.

A functional gene-based molecular screening strategy was used to target type I polyketide synthase (PKS) genes in bioactive fungal endophytes by employing three pairs of degenerate primers. Polyketide synthase gene related to melanin synthase and non reduced polyketide synthase was detected in *Phomopsis* and in three species of *Xylaria*, out of four bioactive endophytic isolates. Bioinformatics analyses of amplified KS domain fragment of polyketide synthase gene facilitated inference of bioactivity of endophytes natural products suggesting that the isolated endophytes produces bioactive secondary metabolites of polyketide origin. The accession numbers obtained for amplified KS domain of PKS fragments were *Xylaria* sp. FPL-10(S) was KC579364 and for *Xylaria* sp. FPL-25(M) was KF147932.

**Fifth chapter** explains the chromatographic and spectroscopic techniques which are employed in the bioassay guided isolation and characterization of antimicrobial metabolites from two potent ethyl acetate extracts of endophytic *Xylaria* viz., FPL-10(S) and FPL-25(M). TLC bioautographic agar overlay assay have been employed throughout chromatographic purification process to track antimicrobial activity. HPLC analysis provided qualitative information regarding ethyl acetate and bioactive fraction obtained from chromatographic purification. LC-hyphenated technique such as LC-MS analysis was used to determine molecular weight of the bioactive metabolites in bioactive fractions identified at *R*$_f$ = 0.45 and *R*$_f$ = 0.78 for *Xylaria* sp. FPL-10(S) and FPL-25(M) respectively. Molecular ion peaks at m/z 181 and 213 in positive and negative ESI-MS mode provide tentative identification of antimicrobial metabolite as a benzoic acid...
derivative and a bis-$\sqrt{\cdot}$-butyrolactones. Spectral data of IR, $^1$H and $^{13}$C NMR analysis supported the identification by providing signals for prominent functional groups, protons and carbon which are responsible for basic chemical backbone of identification of metabolites and identified as 4-cyanomethoxy benzoic acid and Xylobovide 9-methyl ester.

Minimum inhibitory concentrations (MIC) were between 3.13 to 25.0 $\mu$g/ml for Gram-negative bacteria, opportunistic pathogen (Candida albicans) and dermatophytes which is closer to the co-assayed standard drugs nystatin (0.10-3.13 $\mu$g/ml) and gentamicin (0.10-0.78 $\mu$g/ml) for 4-cyanomethoxy benzoic acid metabolite of Xylaria sp. FPL-10(S).

Similarly, minimum inhibitory concentrations were 3.13-100.0 $\mu$g/ml against Gram-negative bacteria, opportunistic pathogen (Candida albicans) and dermatophytes which is closer to the co-assayed standard drugs nystatin (0.10-3.13 $\mu$g/ml) and gentamicin (0.10-0.78 $\mu$g/ml) for xylobovide 9-methyl ester isolated from Xylaria sp. FPL-25(M).

Detection of polyketide synthase gene during genome mining has explained their biosynthetic origin via polyketide pathway.

In conclusion, the data generated in the present study highlighted the seasonal distribution and diversity of endophytic mycoflora from two medicinal plants Mirabilis jalapa and Ficus pumila. Major endophytic fungi isolated were Aspergillus flavus, Petalotiopsis sp., Phomopsis sp. and Xylaria sp. A significant portion of endophytic mycoflora exhibited broad spectrum antimicrobial activity. Ethyl acetate extracts of bioactive endophytic fungi exhibited antimicrobial activity by the production of antimicrobial metabolites such as benzoic acid derivative (4-cyanomethoxy benzoic acid) and a bis-$\sqrt{\cdot}$-butyrolactone (Xylobovide 9-methyl ester) from potent species of Xylaria FPL-10(S) and FPL-25(M). PCR based molecular characterization positively correlated with the detection of polyketide synthase gene in their genome supported the genetic basis of antimicrobial activity. Further work in this direction is necessary to develop a functional gene based screening strategy in the genome mining during bioprospection of endophytic fungal diversity.