6. Summary and conclusion

6.1 Summary

Endophytes are the microorganisms which inhabit inside the living tissues of plants without causing any apparent disease symptoms for their whole life or a short period of span. Among all endophytes, endophytic fungi are considered as one of the most important component of plant micro-ecosystem. Endophytic fungi occupy a unique niche and present in close association with their host plant. So, there is a possibility that they are able to produce a large number of pharmaceutical compounds with great chemical diversity. The plant sources are being extensively explored in search of new biologically active chemical compounds, but endophytic fungi offer a unique and relatively untapped reservoir of bioactive compounds. The present work mainly focuses on the spatial diversity of endophytic fungi associated with *Calotropis procera* and their associated antimicrobial and antioxidant activities. *C. procera* is a well known medicinal plant used for the treatment of various health problems with strong ethnomedicinal history. The systemic exploration of endophytic mycoflora associated with different tissues of *C. procera* may provide information about the plant-microbe relationship and bioactive compounds synthesized by them.

To isolate the endophytic mycoflora associated with *C. procera*, plant samples were collected from 10 geographically different sites, covering five agro-climatic zones of India. The plant material was collected from December 2014 to May 2015. Different tissues (leaf, stem and root) of *C. procera* were screened for the presence of endophytic fungi.

Total 1440 tissues segments (48 segments of each tissue from each collection site) were studied for the isolation of endophytic fungi. Total 578 isolates were obtained from different tissues. These isolates were first identified morphologically and categorized in 26 different morpho-species. Molecular identification was done by ITS primers resulted in the presence of 20 different species belong to 8 genera. The isolated endophytic fungi identified as *Chaetomium atrobrunneum*, *Chaetomium* sp., *Chaetomium arcuatum*, *Penicillium crustosum*, *Penicillium citrinum*, *Fusarium chlamydosporum*,...
Fusarium graminearum, Fusarium solani, Fusarium thapsinum, Fusarium delphinoides, Aspergillus nomius, Aspergillus oryzae, Aspergillus niger, Aspergillus terreus, Aspergillus nidulans, Candida blankii, Curvularia hawaiensis, Cochliobolus hawaiensis, Alternaria alternata and Mucor circinelloides. Majority of fungal isolates were belonged to phylum Ascomycota, except Mucor circinelloides which belongs to phylum Zygomycota.

Aspergillus and Fusarium were the most dominating genus reported in this study. All the sequences obtained by sequencing of PCR products were submitted to NCBI, GenBank database and respective accession numbers were assigned to them.

To find out the diversity pattern of endophytic fungi associated with different tissues of C. procera, colonization frequency percentage (CF %) and different diversity indices were calculated. The overall colonization frequency of endophytes in C. procera was reported as 40.14 %. Aspergillus terreus was the most dominating myco-species (CF % of 5.14 %). Shannon Weiner diversity index (H’) and species richness was reported to be highest for site 8 (Kolkata). This may be because of comparatively higher rainfall as compared to other sites which favors germination and horizontal transfer of endophytic fungi. The higher number of isolates belong to genus Aspergillus (considered as xerophillic fungi) were reported for the plant tissues collected from site 3 (Jhunjunnu) and site 4 (Jodhpur) may be because of their adaptation to flourish in low humid conditions. The Jaccard’s similarity analysis data showed the maximum relatedness between site 5 (Udhampur) and site 7 (Aurangabad) i.e 0.85 while minimum relatedness observed between site 3 (Jhunjhunu) and site 9 (Madhubani) i.e. 0.33.

Among the tissues, maximum number of isolates were obtained from the leaves (257) followed by roots (187) and stems (134) tissues segments respectively. Twenty endophytic fungal species were identified from leaves and roots tissues while, 19 endophytic fungi species were identified to colonize stems tissues. Candida blankii was reported to be absent in the stem tissue. Higher CF % in leaves might be due to the higher surface area of leaves exposed to the environment, presence of various natural openings like stomata, glands etc. Tenderness of leaves as compared to stem and root helps the endophytic fungi to easily colonize the internal tissues.
Colonization frequency of endophytic fungi was significantly influenced by the sample collection sites and tissue types (p<0.0001), while there was no significant variation was reported for species richness, species evenness, Shannon Weiner’s index, dominance ratio and agro-climatic zones as predicted by MANOVA analysis. Presence of dark septate fungi (Alternaria alternata, Cochliobolus hawaiensis, Aspergillus niger, Curvularia hawaiensis) was also reported in the C. procera. Their highest number was reported to present in root tissues and it was found that Alternaria alternata dominates the others.

All the isolated endophytic fungi were screened for their preliminary phytochemical analysis along with their antioxidant and antimicrobial activity. Mass culture of endophytic fungi was done and metabolites of endophytic fungi were extracted with ethyl acetate solvent.

Total phenolic content (measured in terms of gallic acid equivalent) of endophytic fungi was ranged from 16.53±0.27 to 72.71±1.67 µg GAE/mg of fungal extract. Highest TPC was measured in in Aspergillus nomius followed by Fusarium thapsinum (66.08±0.81), Aspergillus niger (59.62±0.13), Penicillium citrinum (59.16±0.05) while Fusarium chlamydosporum was reported to have lowest TPC value. On the other hand, the total flavanoids content of endophytic fungal extracts was ranged from 22.3±0.48 to 49.8±0.75 µg quercetin equivalent/mg of fungal dry weight. Highest TFC content was found in the Aspergillus nomius extract while lowest in Fusarium chlamydosporum.

TAC values were ranged from 70.16±0.67 to 171.33±0.73 µg BE/mg of fungal dry weight. Highest alkaloid content was shown by Aspergillus nidulans while lowest by Chaetomium arcuatum.

The DPPH free radical scavenging activity of different fungal extracts was ranged from 11.17±0.08 to 68.86±0.19%. Highest DPPH free radical scavenging activity was exhibited by Aspergillus nomius while the lowest was shown by Fusarium chlamydosporum.

H₂O₂ free radical scavenging activity was ranged from 18.05±0.03% to 72.19±0.15%, where Aspergillus niger and Fusarium solani showed highest and lowest respectively.
\(\beta\)-carotene-linoleic acid assay was used to assess free radical scavenging activity. It was found that highest free radical scavenging activity by using \(\beta\)-carotene-linoleic acid assay were shown by the extract of *Aspergillus niger* \((60.91\pm0.06\%)\) while, lowest free radical scavenging activity was exhibited by *Candida blankii* \((9.05\pm0.02\%)\).

Highest xanthine oxidase inhibitory activity was exhibited by *Aspergillus nomius* \((67.47\pm0.22\%)\) followed by *Penicillium citrinum* \((66.36\pm0.17\%)\), *Chaetomium arcuatum* \((54.06\pm0.16\%)\) and *Fusarium thapsinum* \((53.59\pm0.16\%)\) while lowest xanthine oxidase inhibitory activity was observed in *Mucor circinelloides* \((19.14\pm0.56\%)\) extracts.

Ethyl acetate extracts of endophytic fungi showed varied degree of metal chelating activity ranged from \(15.13\pm0.02\%\) to \(60.91\pm0.06\%\). Highest metal chelating activity was exhibited by *Aspergillus niger* \((60.91\pm0.06\%)\) while, lowest metal chelating ability was observed for *Mucor circinelloides* \((15.13\pm0.02\%)\).

The principal component analysis (PCA) was performed for the TPC and between different antioxidant assays. A linear positive correlation \((0.165 \text{ to } 0.890, P < 0.05)\) was found between TPC and all the antioxidant assays employed for the antioxidant activity measurement. This relationship represented the homogeneity of results.

Antibacterial activity of crude ethyl acetate extracts of isolated endophytic fungi was evaluated by using agar well diffusion assay against total nine bacterial reference strains. Minimum inhibitory concentration was calculated by microbroth dilution method. Out of total 20 different endophytic fungal extracts, 7 extracts showed antibacterial activity against all tested bacterial strains. Endophytic fungi belong to *Aspergillus* and *Fusarium* genus exhibited good antibacterial activity. Maximum ZOI \((17.33 \text{ mm})\) was shown by *Aspergillus nomius*, *Fusarium solani*, *Aspergillus oryzae* and *Curvularia hawaiensis* against *S. typhi*, *S. flexneri*, *S. typhi* and *S. marcescens* respectively. Extracts of *Aspergillus nidulans*, *Curvularia hawaiensis*, *Chaetomium arcuatum* and *Chaetomium atrobrunneum* also exhibited significant antibacterial activity against the tested bacterial strains. The MIC values were ranged from \(15.6 \mu\text{g/well}\) to \(250 \mu\text{g/well}\). From present analysis, it was found that endophytic fungal
extracts were more efficient against the Gram-positive bacteria than the Gram-negative.

In order to identify the possible active constituents of endophytic fungi, GCMS analysis of ethyl acetate extract of *Aspergillus nomius, Aspergillus terreus, Penicillium citrinum, Aspergillus nidulans, Curvularia hawaiiensis, Fusarium chlamydosporum, Cochliobolus hawaiiensis* and *Mucor circinelloides* was carried out. The compounds present in the crude fungal extracts were reported to exhibit various activities or used in industries for different purposes. Our study revealed the presence of many aliphatic phytoconstituents like linoleic acid, hexadecanoic, ethyl ester, oleic acid, dodecanoic acid, tetradecanoic acid etc. The main metabolites reported were fatty acids, carboxylic acids, and steroids.

Based on the results of antioxidant activity, *Aspergillus nomius* and *Aspergillus terreus* were selected for the synthesis of AgNPs by using their aqueous extracts and 2 mM solution of silver nitrate. Synthesized nanoparticles were characterized by various spectroscopic techniques. TEM analysis of *A. terreus* and *A. nomius* AgNPs showed the spherical and oval particles represented an average size of 16.45 nm and 12.36 nm respectively. Antibacterial activity of synthesized AgNPs was determined against nine reference ATCC bacterial strains and 3 MDR strains. *A. terreus* synthesized AgNPs revealed the ZOI against reference bacterial strains was ranged from 13.67±0.58 to 16.67±0.58 mm. *S. typhi* strain was found to be most susceptible as showed highest ZOI. The MIC of synthesized AgNPs from *A. terreus* against different reference bacterial strains was 11.43-308 μg/ml. The ZOI against MDR bacterial strains was ranging from 13.33±0.58 to 15.33±0.58 mm. *P. aeruginosa* (MDR) strain among all the MDR strains used, was reported to be most susceptible. *K. pneumoniae* (MDR) strain was found to be least susceptible to synthesized AgNPs. MIC values observed for *P. aeruginosa* (MDR) was 34.2 μg/ml, for *E. coli* was 102 μg/ml, for *K. pneumoniae* was 308 μg/ml.

*A. nomius* synthesized AgNPs showed the ZOI against all tested reference bacterial strains. It was ranging from 14.33±0.58 to 17.00±1.00 mm. *S. flexneri* strain was
reported to be most susceptible. The MIC of synthesized AgNPs from *A. nomius* against different reference bacterial strains was 11.43-102.8 μg/ml.

The ZOI for MDR bacterial strains was ranging from 14.00±1.00 to 15.00±1 mm. *K. pneumoniae* (MDR) and *E. coli* (MDR) showed a ZOI of 15.00±1 mm, while *P. aeruginosa* (MDR) strain exhibited 14.00±1.00 mm of inhibition zone. MIC values observed for *P. aeruginosa* (MDR) was 102.8 μg/ml, for *E. coli* and *K. pneumoniae* was 11.43 μg/ml.

During cell leakage analysis, higher protein content was reported in treated cells than control. Highest amount of extracellular protein after treatment with *A. terreus* and *A. nomius* AgNPs was reported for *S. typhi* and *S. flexneri* respectively. Agarose gel electrophoresis showed the degraded band of nucleic acid after application of nanoparticles.

*C. procera* reported to harbour a large diversity of endophytic fungi. Fungal isolated were identified on molecular behalf by using ITS1 and ITS4 pair primer. Highest numbers of isolates were obtained from leaf followed by root and stem tissues. Endophytic fungi isolated from *C. procera* reported to possess considerable antimicrobial and antioxidant activities. GCMS analysis revealed the volatile phytoconstituents present in various fungal extracts. AgNPs synthesized by using *A. terreus* and *A. nomius* exhibited significant antibacterial activity against reference and multidrug resistant strains. These endophytic fungi can be explored further for the isolation and identification of pure bioactive compound solely responsible for these activities.
6.2 Conclusion

- Tissues of *Calotropis procera* were reported to be rich in endophytic fungi population, reported the presence of 20 endophytic fungi belong to the Ascomycota and Zygomycota classes predominately.

- Geographic locations and tissue type significantly influence the colonization frequency of endophytic fungi.

- Crude ethyl acetate extract of isolated fungi found to be rich in various phytochemicals like phenols, flavanoids, alkaloids, saponins carbohydrates etc.

- Crude ethyl acetate extract of endophytic fungi showed significant antioxidant and antimicrobial activity as shown by different assays.

- GCMS analysis of endophytic fungi revealed the richness of extracts in fatty acids, hydrocarbons, carboxylic acids, steroids etc.

- AgNPs synthesized by using *Aspergillus terreus* and *Aspergillus nomius* were showed significant antibacterial activity against reference and MDR strains.
6.3 Significance of work

- The present study provides an extensive view towards the ecology of *Calotropis procera* and endophytic fungal community associated with it.

- Many isolated endophytic fungi showed considerably higher content of secondary metabolites and also reported to possess significant antibacterial and antioxidant activities, which can further be used for the isolation of their potential active compounds for therapeutics.

- Nanotechnology is an emerging field; green synthesis of AgNPs may provide alternative approach to physical and chemical synthesis which possesses greater activity than the crude extracts.
6.4 Future prospectives

- Endophytes showed promising results during phytochemical and antioxidant analysis further need to be studied for isolation of active principal compounds.

- Optimization of culture media and growth conditions for individual endophytes should be performed for production of desire secondary metabolites in greater quantity.

- Further cytotoxic related studies for silver nanoparticle should be carried out to find out the maximum non toxic dose.