CHAPTER- V

DISCUSSION

DISCUSSION

Medicinal plants have been used by ethnic communities all over the globe to treat various ailments. Ethno-medicinal plants from underexplored regions of the world serve as a new ecological niche for a wide variety of endophytes, mostly fungi. However, the potential of endophytic fungi inhabiting medicinal plants for bioactive metabolites remains mostly underexplored in the North-East India, especially Assam. Endophytic fungi inhabit healthy plant tissues without showing any overt incidence of plant diseases. These fungi are highly diverse, may transmit horizontally or vertically and have been found associated in every plant species on the planet. They have been found to confer profound impacts on host plants as mutualists by promoting their growth and tolerance to diseases (Abdel-Azeem *et al*., 2019; Kaur 2020). They have been reported from almost all the plants species studied so far (Chen *et al*., 2011; Khan *et al*., 2013). There is growing interest among researchers to isolate and characterize endophytic fungi inhabiting medicinal plants as they produce a plethora of chemical entities of biotechnological and pharmaceutical significance. In addition, some of them also have the ability to produce the same bioactive metabolites as their host plants (Tan and Zou, 2001; Schulz *et al*. 2002; Gouda *et al*., 2016). The discovery of "taxol" from a host-associated endophytic fungus, have led to the exploration of novel endophytes from medicinal plants since they produce metabolites similar to the host plant (Kusari *et al*., 2014). In the present study, endophytic fungi colonizing healthy leaf tissues of three selected ethno-medicinal plants namely, *Houttuynia cordata* Thunb., *Eryngium foetidum* L. and *Zanthoxylum oxyphyllum* Edgew. were investigated. A total of 214 endophytic fungal isolates were recovered from healthy surface sterilized leaf tissues of the three plant species on different mycological media. The highest recovery of endophytic fungi was found to be in PDA medium. Such findings have also been reported by earlier workers as the most suitable medium for recovery of endophytic fungi (Tayung, 2008; Rodriques *et al*., 2017). However, the maximum number of endophytic fungi from *Z. oxyphyllum* was isolated in modified medium amended with Plant Leaf Extract and Agar (PEA). The addition of host plant extract to the basal medium have been found to show a positive effect on the growth and recovery of endophytes probably because of some additional nutrients specific to the host. Such findings have also been reported by Deka and Jha (2017). The isolated fungi belonged to different classes namely, Ascomycetes, Zygomyecetes, Hyphomycetes and Deuteromycetes were mostly reported as endophytic fungi with the most dominant group isolated being Deuteromycetes. In most of the studies, fungi belonging to the classes Ascomycetes, Deuteromycetes and Basidiomycetes were mostly reported as endophytic fungi (Petrini, 1986; Dayle *et al*., 2001) but the class Deuteromycetes has been found as most dominant endophytes. The result of the present study collaborates with the findings of Khan *et al*., 2010. It was observed that the highest colonizing fungal genera in all the three medicinal plant species was *Colletotrichum* (50%), followed by non- sporulating isolates categorized as Mycelia Sterilia (25.7%) which are represented as "morphotypes" due to their distinct morphological characteristics (Promputtha *et al*., 2005). Other dominant genera isolated were *Purpureocillium* (6.54%) and *Curvularia* (5.61%) as well as the occurrence of other isolates belonging to different genera that include *Corynespora*, *Fusarium*, *Cladosporium*, *Stemphylium*, *Penicillium*, *Scopulariopsis*, *Alternaria*, *Aspergillus*, *Bipolaris* and a dimorphic yeast genus, *Pseudozyma*. The genus *Colletotrichum* and Mycelia sterilia were commonly isolated endophytic fungi from all the three medicinal plants. Similar findings on occurrence of these two groups as dominant endophytic fungi from medicinal plants have also been reported by Photitia *et al*. (2005), Devarajan and Suranarayanan (2006) and Huang *et al*. (2008).

In the recent times, there has been an increasing requirement of alternative sources of antimicrobial drugs to combat the incidence of multidrug resistant microorganisms. Several reports suggest that metabolites produced from endophytic fungi form a major source of novel antimicrobial compounds. Endophytic fungi are therefore considered as a chemical reservoir for novel compounds that possess antimicrobial, anticancer, immunomodulatory, antioxidant, anti-parasitic, antiviral, anti-tubercular and insecticidal properties for use in both pharmaceutical and agrochemical industries (Kaul *et al*., 2012). Therefore, in the present study, an attempt was made to isolate endophytic fungi from some ethno-medicinal plants and to investigate the endophytic fungal isolates for antimicrobial metabolites. The selected ethno-medicinal plants are locally used by the ethnic tribal communities of Assam for the treatment of various stomach related ailments. Reports suggest that the endophytic fungi inhabiting medicinal plants synthesize natural compounds that possess an inhibitory effect against pathogenic microorganisms as well as cure various diseases (Zhao *et al*., 2010). In this study, the crude secondary metabolites obtained from the isolated endophytic fungi were assayed for antimicrobial activity against a panel of clinically significant human test pathogens which are also the causal organisms of stomach ailments. It was found that 78.57% of the endophytic fungal isolates isolated from all the three plants showed antimicrobial activity by inhibiting at least one of the four test pathogens whereas 58.57% of the isolates showed antimicrobial activity against all the test pathogens. Furthermore, 57.14% of the isolates showed antifungal activity and 41.42% of the isolates showed antibacterial activity. The result of antimicrobial assay revealed that from the three selected medicinal plants six endophytic fungal isolates, namely, *Colletotrichum* sp. (HCS3), Morphotype (HCS6), *Scopulariopsis brevicaulis* (EF1), *Purpureocillium lilacinum* (EF6), *Penicillium chrysogenum* (EFB9) and *Colletotrichum gloeosporioides* (ZOB3) showed promising antimicrobial activity against all the clinically significant test pathogens. Earlier studies have also reported that secondary metabolites produced by endophytic fungi belonging to the genus *Colletotrichum* show prominent antimicrobial and cytotoxic activity (Arivudainambi, 2011; Packiaraj *et al*., 2016) which is also reflected in the present study. Additionally, in the present study, several non-sporulating fungi categorized as Mycelia sterilia showed promising antimicrobial activity. The occurrence of non-sporulating fungi as endophytes with antimicrobial activity has been reported from several plant species (Padhi and Tayung, 2015). Secondary metabolites of endophytic fungi *Purpureocillium lilacinum*, *Penicillium chrysogenum* and *Scopulariopsis brevicaulis* are also reported to show inhibitory effects against various human pathogens as also observed in our study (Devi *et al*., 2012; Lenta *et al*., 2016; Rashmi *et al*., 2019).

The production of antimicrobial metabolites is often influenced by nutritional as well as environmental factors and is considered as one of the significant parameters for industrial production. Factors like pH, temperature, incubation period and type of media have been reported to effect the production of bioactive metabolites (Thakur *et al*., 2009). In various instances, cultural conditions and nutritional optimization have significantly found to influence the production of secondary metabolites (Bhattacharyya and Jha, 2011). Therefore, in this study, the endophytic fungi that showed promising antimicrobial activity were grown in different nutritional and environmental conditions for enhanced antimicrobial metabolite production. Most of the isolated fungi showed enhanced antimicrobial activity when grown under different cultural conditions. The results showed that maximum antimicrobial activity against the test pathogens was shown by *Colletotrichum* sp. (HCS3), Morphotype (HCS6), *Scopulariopsis brevicaulis* (EF1), *Colletotrichum gloeosporioides* (ZOB3) and *Penicillium chrysogenum* (EFB9) when grown in PDB medium. This corresponds with the earlier studies that suggest that cultivation of fungi in PDB medium enhances antimicrobial metabolite production (Seephonkai *et al*., 2001; Gupte and Kulkarni, 2002). Similarly, enhanced secondary metabolite production was also observed in CDB medium in *Purpureocillium lilacinum* (EF6). The low amounts of glucose in CDB medium have been reported to enhance activity for some fungus (Hutter, 1982). In addition, CDB medium also contains nitrogen sources and other inorganic compounds apart from carbon sources that might affect the enhancement of antimicrobial activity. Many workers have reported that the incubation periods of 7 days (Lin *et al*., 2005), 14 days (Gasong and Tjandrawinat, 2016) and 21 days (Hegge *et al*., 2015) yield maximum antimicrobial metabolites. In this study, most of the isolates showed maximum inhibitory activity in 14^{th} day while some showed optimum production in 21^{st} day. However, no isolate showed maximum activity when incubated for 7 days which contradicts with some earlier reports. Four of the most potent isolates, namely, *Colletotrichum* sp. HCS3, *Scopulariopsis brevicaulis* EF1, *Penicillium chrysogenum* EFB9 and *C. gloeosporioides* ZOB3 showed optimum antimicrobial metabolite production under neutral pH which is similar to the reports by several other workers (Gogoi *et al*., 2008; Padhi and Tayung, 2013; Kaur and Arora, 2020). Although there are very few reports on production of optimum secondary metabolites under alkaline pH (Zhao *et al*., 2010), two of the isolates, Morphotype HCS6 and *Purpureocillium lilacinum* EF6 showed enhanced inhibitory activity under alkaline pH that indicates their alkalophilic nature. Results regarding changes in incubation temperature indicated that all of the isolates showed maximum antimicrobial activity at temperature of 30^{o}C. None of the isolates showed better activity when cultivated in higher temperatures of 35^{o}C and 40^{o}C respectively. This reflected the non-thermostable nature of the metabolites and their inability to either thrive or produce bioactive metabolites at higher temperatures.

Molecular tools including phylogenetic analyses along with the traditional techniques have been proven to be successful for correct identification of fungi and have revolutionized the fungal classification system (Sarwar *et al*., 2019). Therefore, two of the endophytic fungi with promising antimicrobial activity, namely, *Colletotrichum* sp. (HCS3) and Morphotype (HCS6) were identified through molecular technique using ITS rDNA sequence analysis. ITS region which consists of a highly conserved 5.8S rRNA and variable regions ITS1 and ITS2, is one of the widely used phylogenetic marker for fungal species identification (Tamura *et al*., 2004; Xu and Adamowicz, 2016). The sequences were confirmed and phylogenetic relationships were determined by ITS2 sequence phylogeny and RNA secondary structure analysis. In addition, recent reports revealed the sequence variability in ITS2 is more appropriate for phylogenetic reconstruction and species differentiation in eukaryotes as well as in fungi and can be improved further if structural features are taken into account (Marinho *et al*., 2011; Heeg and Wolf, 2015; Zhang *et al*., 2020). In the present study, homology search and phylogenetic analysis using ITS as well as ITS2 sequence of the *Colletotrichum* sp. (HCS3) revealed it to be *Colletotrichum coccodes*. Furthermore, comparison of the ITS2 RNA secondary structures revealed its close affinity with endophytic *C. coccodes*. However, it showed differences with pathogenic *C. coccodes* and displayed significant variations in terms of folding pattern and motif structures. Similarly, the homology search and phylogenetic analysis using both ITS and ITS2 sequence of the isolate Morphotype

(HCS6) revealed it to be *Phyllosticta capitalensis*. The ITS2 RNA secondary structure of the isolate HCS6 showed that it shared a close affinity with an endophytic *Phyllosticta capitalensis*. However, due to unavailability of complete ITS2 sequences of pathogenic *P. capitalensis* comparison could not be drawn with their pathogenic lifestyle. Similar observations have also been put forth by several studies (Padhi and Tayung 2013; Padhi *et al*., 2016) which suggested ITS2 secondary structure could be used as a phylogenetic marker to establish similarities and variations between different lifestyles of same species.

The crude secondary metabolites extracted from all the six potent endophytic fungi were characterized using different analytical and spectroscopic techniques. FTIR and GCMS analyses of the endophytic fungi revealed the presence of different functional groups and the identified compounds were found to have bioactive properties. FTIR analysis of the metabolites obtained from endophytic fungus, *Colletotrichum coccodes* HCS3 revealed presence of ester and δ- lactones classes of compound. These compounds have been reported to have antimicrobial and antitumor properties (Yixi, 2015). Further, the metabolites also showed presence of Sulphonamides and Nitro groups that are reported as effective antimicrobial agents (Tačić *et al*., 2017; Andrade *et al*., 2020). Further, the crude extract of *C. coccodes* was also characterized by GCMS analysis and the chromatogram so obtained showed presence of several bioactive metabolites. One of the promising metabolite that was identified in the extract was Geranyl geraniol. This metabolite has been reported to show antimicrobial activities especially against *S. aureus* (Kobayashi, 2005). Therefore, it can be attributed that the antibacterial activity against *S. aureus* of the crude metabolite in the present study may be presence of this compound. Another compound, Farnesol was also detected in the crude extract. Interestingly, this metabolite has been reported to be an anti-quorum sensing molecule and showed inhibitory effect against *C. albicans* (Derengowski *et al*., 2009). Further, another compound like Squalene was also identified in the extract. This compound has been reported to be used in the treatment of wounds and skin diseases. Besides, the compounds also showed antioxidant and cytotoxic activities against various human cancer cell lines (De Los Reyes *et al*., 2015). Other compounds include Phenol, 2, 4-Bis (1, 1-Dimethyl ethyl) that have been reported to show good antioxidant activity (Padmavati, 2014). Oleic Acid, another identified compound is widely known to have anticancer effects in many tumors and anticancer effect against tongue squamous cell carcinoma (Jiang *et al*., 2017). Interestingly, compounds like Undecanoic acid and N-Hexadecanoic acid that are reported in plant extract of *H. cordata* (Liang, 2005) was also detected in the extract obtained from endophytic fungus *C. coccodes*. Such results support the view that novel endophytes from medicinal plants produce metabolites similar to the host plant (Kusari *et al*., 2014). Moreover, the compound Undecanoic acid is known to be an antifungal agent and also inhibits bio-film formation (Shi et. al, 2016). Similarly, N-Hexadecanoic acid is reported to be anti-inflammatory in nature (Aparna *et al*., 2012). This supports the assumption that the antimicrobial effect of the medicinal plant might be because of the bioactive secondary metabolites produced by endophytic fungi or vice-versa (Stierle *et al*., 1993; Zhao *et al*., 2011). Similar analysis of ethyl acetate extracts of endophytic fungus *Phyllosticta capitalensis* HCS6 revealed presence of amines, α, β-unsaturated esters, esters and δ-lactone which are reported to have antimicrobial properties (Andrade *et al*., 2020; Yixi, 2015). Other compound classes in the extract include alkenes, alkanes, aromatic esters and alkyl aryl ether. Again, GCMS analysis of the extract of *P. capitalensis* indicated the presence of compounds like 7-Hexadecene, 7-Tetradecene, Undecanoic Acid, 2-Decanynoic Acid, 1-Octacosanol, 9-Tricosene, 8-Heptadecene, Acetic Acid, Hexadecyl Ester and 5-Eicosene. All these compounds have been reported to exhibit various bioactive properties. Out of these, a major compound, 1-Octacosanol presence in the extract has been reported to be good anti-oxidant and antibacterial agents (Sengupta *et al*., 2018). Other identified compounds in the extract, namely, Phenol, 2, 4-Bis (1, 1-Dimethylethyl), Oleic Acid, Phenol,3,5-Bis (1,1-Dimethylethyl) and Hexacosanol Acetate have been reported to exhibit antimicrobial, anti-inflammatory and antitumor properties (Mbosso *et al*., 2010; Padmavati, 2014; Jiang *et al*., 2017). FTIR spectroscopic analysis of ethyl acetate extracts of another potent endophytic fungus *Scopulariopsi brevicaulis* EF1 revealed presence of different functional groups like alkanes, aromatic esters, alcohol, primary amines, including compound classes like esters and δ-lactones. Esters and δ-lactones are reported to have antimicrobial properties (Yixi, 2015). GCMS analysis of the crude extracts of the fungus *S. brevicaulis* revealed presence of several bioactive compounds like Phenol, 2, 4-Bis (1, 1-Dimethyl ethyl) and Oxirane (Padmavati 2014; Montana *et al*., 2014). Another pharmaceutical excipient namely, Acety tributyl citrate was also identified in the extract that has been reported to have antimicrobial properties (Kim *et al*., 2018). Similarly, study of ethyl acetate extracts of endophytic fungus *Purpureocillium lilacinum* EF6 also revealed presence of different compound classes belonging to Mono-substituted benzene derivatives, tertiary alcohols, alkyl aryl ether, alkanes as well as esters and δ-lactones that are reported have antimicrobial properties (Yixi, 2015). The GCMS chromatogram revealed presence Dibutyl phthalate and 1, 2-Benzene dicarboxylic acid which have been reported as antimicrobial agents (Roy *et al*., 2006; Garba *et al*., 2016). Further, FTIR spectrum of the ethyl acetate extract of endophytic fungus, *Penicillium chrysogenum* EFB9 also revealed presence of various functional groups containing compound classes that include esters, tertiary alcohols, fluoro compounds, phenols, alkane (methyl group), alkenes, alcohols and primary amines. GCMS study of the extract indicated presence of Dibutyl phthalate, Tributyl acetyl citrate and 1, 2-Benzene dicarboxylic acid that have been reported to have antimicrobial activities (Roy et al., 2006; Roy et al., 2016; Kim et al., 2018). Compounds like Geranyl geraniol and Squalene were also obtained in the crude extract. As reported earlier these compounds have antimicrobial, antioxidant and cytotoxic against cancer cell lines (Kobayashi, 2005; De Los Reyes *et al*., 2015). Bioactive compounds Hexadecanoic acid and Tetradecanal were also revealed to be present in the fungal extract of *P. chrysogenum* which are reported to possess antifungal, antioxidant and anti-cancer properties (Mujeeb *et al*., 2014; Devi and Serfoji, 2018). These compounds have also been reported to be present in the leaves of *E. foetidum* (Nataraj *et al*., 2020). This indicates that the endophytic fungi have either developed the ability to produce the same bioactive substances as those of their host plants or the therapeutic properties of the plant might also be the result of the bioactive metabolites produced by the endophytes as already suggested by Stierle *et al*. (1993) and Zhao *et al*. (2011). The FTIR analysis of crude ethyl acetate extract obtained from endophytic fungus, *Colletotrichum gleosporoides* ZOB3 also revealed presence of different functional groups such as esters, tertiary alcohols, fluoro compounds, nitro compounds, phenols, alkanes (methyl group), alkenes, alcohols and primary amines. GCMS analysis of the extract revealed presence of compounds like 2-Allylphenol, Oxirane and 3-Methyl-2-(2-Oxopropyl), Furan that are reported to be antioxidant, antimicrobial, anti-proliferative and anti-inflammatory (Neto *et al*., 2019; Montana *et al*., 2014; Al-Wathnani *et al*., 2012; Biswal, 2020). Endophytic fungi have been recognized as prolific producer of bioactive metabolites. Recently, fungal endophytes isolated from medicinal plants have demonstrated wide array of biologically active molecules with multiple applications. Such assumption is well documented in this present study, where FTIR and GCMS analyses of the metabolites obtained from endophytic fungi isolated from medicinal plants revealed variety of bioactive compounds with prominent antimicrobial activities against bacterial and fungal test organisms. Some of the compounds like Geranyl geraniol, Squalene, Phenol,2,4-Bis (1,1-Dimethyl ethyl), Dibutyl Phthalate, Oleic Acid, 1-Octacosanol, Undecanoic acid and Hexacosanol Acetate which were reported to exhibit antimicrobial, antioxidant, anti-inflammatory and cytotoxic activities were obtained from *C. coccodes*, *P. capitalensis* and *P. chrysogenum*. However, antimicrobial compounds like Undecanoic acid and N-Hexadecanoic acid which were already reported in leaf extracts of *H. cordata* were also revealed in the crude extract analysis of *C. coccodes* isolated from the leaves of *H. cordata* in this study. Similarly, bioactive compounds Hexadecanoic acid and Tetradecanal were also revealed to be present in the fungal extract of *P. chrysogenum* isolated from *E. foetidum* which has also been reported to be present in the leaves of *E. foetidum*. Amongst the isolates, metabolites obtained from *C. coccodes* showed prominent antimicrobial activity against the test organisms. Therefore, the organic crude extract obtained from this fungus was purified using bioassay guided fractionation. Characterization of the active fraction by NMR data revealed a bioactive polyphenolic compound identified as tyrosol. Tyrosol is a well-known secondary metabolite produced by both plants (Capasso *et al*., 1992) and fungi including *Diplodia seriata* (Venkatasubbaiah and Chilton, 1990), *Alternaria tagetica* (Gamboa-Angulo *et al*., 2001), *Neofusicoccum parvum* (Evidente *et al*., 2010) and *Neofusicoccum australe* (Andolfi *et al*., 2012), *Lasiodiplodia* spp. (Cimmino *et al*., 2017), *Diaporthella cryptica* (Cimmino *et al*., 2018). Moreover, several studies have also reported tyrosol as a bioactive metabolite from crude extracts of endophytic

Colletotrichum (*C. gleosporoides* and *C. crassipes*) (Anisha and Radhakrishnan, 2017; Masi *et al*., 2018; Plontikov and Plontikova, 2020). However, report of tyrosol from *C. coccodes* is limited. Interestingly, in the present study tyrosol isolated from an endophytic *C. coccodes* extract showed significant antimicrobial activity. Tyrosol is a polyphenol that is reported to possess antimicrobial and antioxidative properties and also inhibit biofilm formation (Abdel-Rhman *et al*., 2015; Casadey *et al*., 2020). Tyrosol isolated from marine fungi have been reported to show anti-Quorum Sensing effects by inhibiting the growth of pathogenic microorganisms (Chang et al., 2019). So far, this is the first report of isolation of this compound from endophytic fungus, *C. coccodes*. Further, Tyrosol as well as Farnesol revealed by GCMS analysis have also been reported as Quorum Sensing (QS) inhibiting molecules that regulate the cell-cell communication and the transition between cells based on their density. Both the molecules have an antifungal effect that needs further research (Rodrigues and Černáková, 2020).

The present work therefore suggests that endophytic fungi inhabiting ethno-medicinal plants prove to be a good source of antimicrobial metabolites. Considering the myriad of the medicinal plants used by the tribal communities of Assam, further studies might result into the discovery of potent fungal strains with varied therapeutic applications. Therefore, to combat the merging incidence of drug resistance, research priorities could be directed to study endophytic fungi colonizing traditionally used medicinal plants of unexplored biodiverse regions. Also, in addition to isolation of endophytes, RNA secondary structures could be used as a promising tool to establish the variations and similarities among different lifestyles of fungi and its endophytic counterpart, as indicated in this study.