Research Article

Endophytic Fungi Associated with Traditional Medicinal Plants of Manipur

Nameirakpam Nirjanta Devi*, Mutum Shyamkesho Singh
Department of Life Sciences, Manipur University, Chanchipur, Manipur, India.
***Corresponding author's E-mail:** nnirjan@gmail.com

Accepted on: 13-05-2015; **Finalized on:** 30-06-2015.

ABSTRACT

In this study Endophytic fungi associated with 10 medicinal plants of Manipur have been investigated. 211 endophytic fungi were classified by using morphological method. All the plant samples were found to harbour various endophytic fungi with different colonization frequency (CF) and isolation rates (IR). *Centella asiatica* Linn and *Phlogacanthus thyrsiflorus* Nees shows highest IR value whereas *Allium odorum* Linn, has lowest IR value (0.36) and CF value (66.66). Mycelia sterilia was most ubiquitous endophytic fungal found in this study. Mycelia sterilia consists of various morphological fungal types, but not forming spores. In addition *Alternaria* sp., *Aspergillus* sp., *Colletotrichum* sp., *Chaetomium* sp., *Cladosporium* sp. were dominant endophytic fungi. The Minimum inhibitory concentration (MIC) values for the fungal extracts ranged from 25.0µg/ml to 100µg/ml. Free radical scavenging activities of ethyl acetate extract of *Alternaria* sp. showed the highest scavenging property. This study shows that traditional medicinal plants of Manipur are rich source of endophytic fungi.

Keywords: Biodiversity, Endophytic fungi, Manipur, Medicinal Plants.

INTRODUCTION

Endophytes are microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects. Endophytes are important components of microbial diversity. There has been a great interest in endophytic fungi as potential producers of novel, biologically active products[1,2].

A medicinal plant may be defined as any plant which in one or more of the organ contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs. Plants with ethnopharmaceutical importance are being exploited because of their healing properties.

Medicinal plants provide a unique environment for endophytes and have been recognized as a repository of endophytes with novel metabolites of pharmaceutical importance[3,4].

The popular medicinal plants that have an ethnobotanical history were selected for investigation of endophytic fungi.

The selected plant species and their ethno-medicinal records[5] are given below:

***Phlogacanthus thyrsiflorus* Nees (Acanthaceae)**

Shrub, wild/cultivated; F/F- June – September; fresh shoot and leaf cooked in water and the soup is taken for cough and fever. Inflorescence is eaten raw along with salad; plant part especially leaf are boiled in water and consumed the liquid in stomach ulcer, intestinal disorder and muscular sprain.

Centella asiatica Linn (Apiaceae)

Prostate herb, found round the year, whole plant parts are used; extract is given in cold, cough, dyspepsia and used as health tonic.

***Gynura angulosa* De (Asteraceae)**

Tall succulent herb; Wild, very common in wet places in valley as well as hills upto medium altitude; F/F- Round the year; Leaf/shoot decoction is applied in fresh cut and injuries which stop bleeding.

Fresh leaf and shoot are cooked and the soup is taken a glassful after food everyday for a week to cure stomach ulcer.

***Ageratum conyzoides* Linn (Asteraceae)**

Annual hispidate aromatic herb; F/F – Oct-Feb; Wild; very common in the valley areas especially swampy places as well as low altitude hills; Leaf extract is applies in fresh injuries.

Fresh shoot and leaf is used as a gradient in the preparation of traditional hair lotion locally called as Chingee.

***Eupatorium birmanicum* De (Asteraceae)**

Bushy undershrub; Cultivated/Wild in valley areas especially near foothills; F/F- Nov-March; Fresh leaf or shoot decoction paste is applied on forehead in excessive body temperature and in severe fever.

***Houttuynia cordata* Thunb (Saururaceae)**

Herb; Wild/Cultivated; F/F-Jul-Oct; Leaf extract given in dysentery, boil extract of the plant is given in muscular sprain.

***Allium hookerii* (Linn) (Liliaceae)**

Spicy herb; F/F-Sept-Dec; Cultivated; Leaf decoction paste is applied on excessive body temperature and vertigo.

***Allium odorum* Linn (Liliaceae)**

Aromatic herb; F/F- Sept-Dec; Cultivated; Plant soup after boiling with water is taken against urinary disorder.

***Plantago major* Linn (Plantaginaceae)**

Herb; Wild; F/F-Jun-Feb; Seed powder paste eaten in fever. Plant is semi boiled with litter water and the material is applied in boils, muscular sprain and gout.

***Mimosa pudica* Linn (Mimosaseae)**

Straggling spiny undershrub; Wild; F/F-Round the year; Leaf extract is taken in hydrocele, piles, boils and in jaundice.

MATERIALS AND METHODS

Collection of Plant Materials

A total of 10 medicinal plants were selected for this work. Medicinal plants used in this research include 10 species from 7 families. The samples from healthy living medicinal plants in Imphal West used for isolation of endophytic fungi were collected in different places from January to September, 2013. All the fresh samples were taken to the laboratory and treated within 24 hr.

Isolation of Endophytic Fungi

The plant materials were rinsed gently with running tap water. The leaves (5X5mm) stem and roots (10mm in length) were cut into pieces. The isolation of endophytic fungi followed a modified procedure[6]. All segments were successively surface- sterilized by dipping in 70% ethanol for 1 min, 2.5% sodium hypochlorite for 10 min, and again 75% ethanol for 1 min, followed by rinsing in sterile water three times. The plant pieces were blotted in sterile blotting paper. In each petri dish, a total of 4-6 processed segments were evenly placed on PDA medium supplemented with 200 µg/ml streptomycin, followed by incubation at 28°C until the mycelium or colony originating from the surface of the segments appeared. Hyphal tips were transferred and cultured on potato dextrose agar (PDA) plates.

Data Analysis

Colonization Frequency (CF) were calculated as the total number of segments colonized by endophytic fungi divided by the total number of segment observed and they were expressed as percentage[7]. Isolation rates were calculated as the number of isolates obtained from segments, divided by the total number of segments, but not expressed as percentage. Isolation rates were a measure of fungal richness in a given sample of plant tissue. Relative frequencies of isolation was calculated as the number of isolates of one species were divided by the total number of isolates, and expressed as percentage[8].

Identification of Endophytic Fungi

The morphological identification of the endophytic fungal strains was based on the morphology of the fungal culture colony, hyphae, the characteristics of the spores, and the reproductive structures[9,11]. For sporulation, the fungal isolates were inoculated on PDA. Slide culture methods was used for fungal identification. Morphological characteristics of conidiogenous cells and conidia of all fungal characters were made in water mounts, and the slides were subsequently mounted in lactophenol and sealed with nail vanish. All experiments and observations were repeated at least twice. Those cultures which failed to sporulate were named as mycelia sterilia, and divided into different morphospecies according to their cultural characteristics.

Cultivation of Endophytic Fungi

The fresh mycelia of selected endophytic fungi from medicinal host plants were inoculated in 500ml flask containing 200ml potato dextrose broth followed by incubation with a shaking incubator at 140 rpm for 30 days at 28°C. The culture broth of each endophytic fungus was filtered by Whatmann Filter paper to remove mycelium.

Extraction of Metabolites

Metabolite was extracted by solvent extraction procedure using ethyl acetate as organic solvent. Equal volume of the filtrate and ethyl acetate was taken in a separating funnel and shaken vigorously for 10 min. The samples were extracted three times with ethyl acetate. Ethyl acetate collected after extraction was evaporated and the resultant compound was dried in vacuum evaporator using $MgSO_4$ to yield the crude metabolite. After evaporation a brown coloured crude extract was obtained. The crude extract was then dissolved in Dimethyl sulphoxide (DMSO) until analysis.

Bioassay of Endophytic fungi against Plant Pathogenic Fungi

Bioassay of endophytic fungi against plant pathogenic fungi was done by dual culture technique[12]. Test pathogenic fungi include *Fusarium* oxysporum and *Trichoderma viride*.

Antibacterial Assay

Extracts from endophytic fungi were screened for their antibacterial activity against human pathogenic bacteria using agar well diffusion method. Test Bacterial strain include two Gram Positive Bacteria- *Staphylococcus aureus*(ATCC25923), *Enterococcus faecalis* (ATCC29212) and two Gram negative bacteria- *Escherichia coli* (ATCC25922) and *Pseudomonas aeruginosa* (ATCC27853). About 1ml of the inoculums of the test pathogen was spread into Muller Hinton Agar plates. A 5mm well was made in each corner of the plate with equal distance using a sterile cork borer. The ethyl acetate extract with different concentrations at 25, 50, 75 and 100µg compared with standard antibiotic were placed in their respective well and the plates were incubated at 37°C for 48h. DMSO was used as a control. After the incubation, the inhibition zone around the well was recorded and expresses as millimetre.

Determination of free radical scavenging activity

The free radical scavenging activity was determined by DPPH method[13]. DPPH radical scavenging assay were performed to study the antioxidant potency of extract of endophytic fungi. Each sample (3mL at 0.025g/ mL) was mixed with a DPPH solution (45µg/ mL, Sigma) in HPLC grade methanol (Merck), vortexed well at room temperature and left standing exactly for 10 min. The UV/VIS absorbance was measured at 517 nm serving the methanol without DPPH solution as blank solution. A reference solution (125µg/ mL) of Butylated hydroxyl toluene (BHT, Sigma) in methanol was used taking 100% radical scavenging activity. The scavenging percentage was calculated using the equation:

$$\%\,Scavenging\ = (A_0 - A_5) \times \frac{100}{(A_0 - A_5)}$$

where, A_0, A_5 are the absorbance values of DPPH + Sample solution at 0.0 min and after 0.5 min, respectively; A_0, A_5 are the absorbance values of DPPH + BHT at 0.0 min and after 0.5 min.

Determination of total phenolic content

Dilutions of Sample (0.5ml) were oxidised with 2.5mL Folin-Ciocateau for 5 minutes at room temperature. Then the reaction was neutralized with 2mL Sodium Carbonate. The absorbance of the resulting blue colour was measured at 650nm with the spectrophotometer after incubation for 2hr at room temperature in dark. Quantification was done on the basis of the standard curve of gallic acid. All the test were carried out in triplicate. Results were expressed as gallic acid equivalent (GAE), i.e., mg gallic acid/g of extract.

Statistical Analysis

All sample determinations were conducted in triplicates and the results were calculated as mean ± standard deviation (SD) in this study. Coefficients of determination (R^2) were calculated using Microsoft Excel 2007.

RESULTS

Isolation of Endophytes

A total of 211 endophytic fungal strains were isolated from the 10 traditional medicinal plants of Manipur. All the plant samples were found to harbour various endophytic fungi with different colonization frequency (CF) and isolation rates (IR) (Table 1). *Centella asiatica* Linn and *Phlogacanthus thyrsiflorus* Nees shows highest IR value whereas *Allium odorum* Linn, has lowest IR value (0.36) and CF value (66.66). The mean CR value of these 10 medicinal plants was 80.13 and their mean IR value was 0.65.

Table 1: Endophytic fungal colonization frequency and isolation rates in 10 traditional medicinal plants

Species	Number of samples	Number of samples yielding isolates	Number of isolates	Colonization frequency (%)	Isolation rates
Centella asiatica Linn (L/S)	30(20/10)	23(15/8)	25(23/2)	76.66	0.83
Phlogacanthus thyrsiflorus Nees(L)	30	21	23	70.00	0.76
Gynura angulosa De(L)	40	36	26	90.00	0.65
Ageratum conyzoides Linn	30	24	24	80.00	0.80
Eupatorium birmanicum De	36	27	30	75.00	0.83
Houttuynia cordata Thunb	20	19	14	95.00	0.70
Allium hookeri	40	34	22	85.00	0.55
Allium odorum Linn	36	24	13	66.66	0.36
Plantago major Linn	30	24	18	80.00	0.60
Mimosa pudica Linn	30	25	16	83.33	0.53

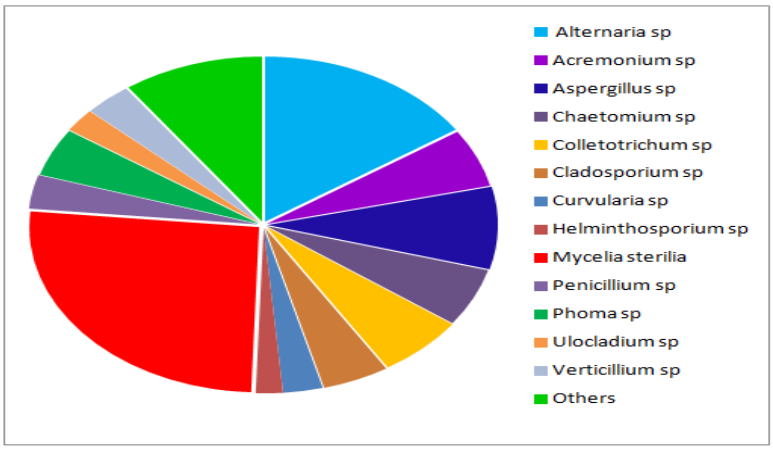

Figure 1: Relative frequencies of different endophytic taxa totally isolated from 10 medicinal plants of Manipur.

Table 2: The number and taxonomic identification of endophytic fungi isolated from 10 medicinal plants of Manipur

Plant	Al	Ac	As	Ch	Co	Cl	Cu	He	Ms	Pe	Ph	Ul	Ve	Others
Centella asiatica Linn (L/S)	8		2	4				2	4	2				3
Phlogacanthus thyrsiflorus Nees (L)	4		2		6	2	2		5		1	3		2
Gynura angulosa De (L)	3	7		2		3	2		7	2		1	2	2
Ageratum conyzoides Linn			4			2			7		2			3
Eupatorium birmanicum De	4	1			3		1		7	1			2	2
Houttuynia cordata Thunb	3	3	4	3				2	4	1	2		3	
Allium tuberosum Roxb/hookeri						2	1		4		3			3
Allium odorum Linn	4		2		2				6	1				
Plantago major Linn		1		4		1			7		2	1		2
Mimosa pudica Linn	6		3		2				4					4
Total	32	12	17	12	13	10	6	4	55	7	10	5	7	21
Overall isolates	211													

Al: *Alternaria* spp. (3 morphospecies); Ac: *Acremonium* sp; As: *Aspergillus* spp. (2 morphospecies); Ch: *Chaetomium* sp (3 morphospecies); Co: *Colletotrichum* spp. (4 morphospecies); Cl: *Cladosporium* sp.(2 morphogenesis); Cu: *Curvularia* sp; He: *Helminthosporium* sp; Ms: Mycelia sterilia spp.; Pe: *Penicillium* sp; Ph: *Phoma* sp; Ul: *Ulocladium* sp; Ve: *Verticillium* sp

Table 3: Endophytic fungal isolates showing antibacterial activity

Endophytes	Zone of inhibition(mm)			
	Staphylococcus aureus	*Enterococcus faecalis*	*Escherichia coli*	*Pseudomonas aeruginosa*
***Alternaria* sp 2**	14.33 ± 1.52	9.6 ± 0.50	11 ± 0.5	15.3 ± 0.5
***Helminthosporium* sp**	13 ± 1.00	8.3 ± 0.6	-	9 ± 1
Colletotrichum gloeosporioides	13.33 ± 1.52	-	9 ± 1.1	11.3 ± 1.5
Mycelia sterilia 2	11 ± 0.57	7.3 ± 0.5	-	11 ± 2.5
***Curvularia* sp**	12.33 ± 1.52	-	10.3 ± 1.5	10.6 ± 1.1

Identification of Endophytic Fungi

The 211 endophytic fungal strains isolated from 10 medicinal plants were classified into 13 taxa based on their characteristics of culture colony and reproductive structure (Table 2). Mycelis sterilia showed the highest relative frequency (RF: 26%).

Mycelia sterilia was most ubiquitous endophytic fungal found in this study. Mycelia sterilia consists of various morphological fungal types, but not forming spores. In addition *Alternaria* sp (15.16%), *Aspergillus* sp (8.05%), *Colletotrichum* (6.16%), *Chaetomium* sp (5.7%), *Cladosporium* sp (4.7%) were dominant endophytic fungi as in Figure 1.

Antimicrobial Activity

The crude metabolites of most of the endophytic fungi isolated from the medicinal plants displayed considerable antibacterial activity against the test pathogenic bacteria.

The crude extract of five endophytic fungi *Alternaria* sp 2, *Helminthosporium* sp, *Colletotrichum gloeosporioides*, Mycelia sterilia 2 and *Curvularia* sp showed effective inhibition against the test bacterial strain (Table 3).

The Minimum inhibitory concentration (MIC) values for the fungal extracts ranged from 25.0µg/ml to 100µg/ml.

The result against plant pathogenic fungi by dual culture method was not satisfactory.

Antioxidant Capacity

Free radical scavenging activities were measured by DPPH method. Using this method antioxidant capacity of the endophytic fungal isolates from medicinal plants were assessed.

All the samples were found to possess scavenging property. Free radical scavenging activities of ethyl acetate extract of *Alternaria* sp 2 showed the highest scavenging property (95%).

Total Phenolic Content

The total phenolic content of the 15 selected endophytic fungal cultures were estimated using the Folin-Ciocalteu colorimetric method. The total phenolic contents of the endophytic fungi are given in Table 4. *Alternaria* sp 2 showed the highest total phenolic content (19mg gallic acid/g of extract), followed by *Colletotrichum gloeosporioides* (18mg gallic acig/g of extract).The total antioxidant activity of the endophytic fungal metabolites was significantly correlated with their total phenolic content.

Table 4: Total content of phenolics in metabolites from 15 selected endophytic fungi

Fungal taxon(Code)	TPC(mg gallic acid/g of extract)
Penicillium sp(C1)	9.4 ± 1.46
Chaetomium sp(C2)	7.0 ± 0.04
Mycelia sterilia 2(C3)	17.36 ± 0.88
Alternaria sp 1(C4)	8.96 ± 0.45
Alternaria sp 2(C5)	19 ± 0.10
Mycelia sterilia 1(C6)	16.91 ± 0.39
Helminthosporium sp(C7)	17.43 ± 0.28
Phoma sp (N2)	8.43 ± 0.38
Curvularia sp(N3)	17.07 ± 0.83
Cladosporium sp (N4)	11.72 ± 0.23
Aspergillus sp(N5)	13.02 ± 0.33
Acremonium sp(L1)	16.43 ± 0.146
Verticillium sp(L2)	8.3 ± 0.21
Ulocladium sp(T1)	14.80 ± 1.50

DISCUSSION

In this study all the medicinal plant investigated were found to harbor endophytic fungi. 211 endophytic fungi were isolated in this study. The quest for finding new bioactive compounds paves the way to study endophytic fungi. Endophytic fungi have been widely investigated for novel bioactive compounds. Endophytic fungi have been studied from limited plants. Studies are focused on endophytic fungi associated with medicinal plants for their bioactive compounds. Endophytic fungi have been recognized as repository of novel secondary metabolites. Endophytes are considered to be metabolically more active than their free counterparts due to activation of various metabolic pathways to survive inside the host tissues. Medicinal and endemic plants should be use for endophytic studies as they harbor useful endophytes with novel metabolites . Studies are reported where native medicinal plants are used to screened fungal endophytes[14,15]. Mycelia sterilia, *Alternaria* sp., *Curvularia* sp., and *Chaetomium* sp., were the frequently isolated endophytes, Mycelia sterilia has been reported as endophytes in several studies[16]. Based on morphological identification two hundred and eleven isolates were obtained from ten medicinal plants of Manipur. Screening for antimicrobial compounds from endophytes is an promising step to overcome the issues related with drug reisistant microbes. Previous studies indicated that bioactive metabolites from endophytic fungi possessed antimicrobial property[17]. The antioxidant capacities of endophytic fungi from Chinese medicinal plants were investigated[18].

CONCLUSION

This preliminary study of screening endophytic fungi from medicinal plants of Manipur shows the diversity of endophyte.

Our study supports the previous finding that endophytic fungi are highly available in the medicinal plants.

Further work may yield potent compounds with various application. In conclusion, endophytic fungi from medicinal plants of Manipur could represent a potent source of bioactive compounds.

ACKNOWLEDGEMENT

This work was supported by Department of Biotechnology, Government of India.

REFERENCES

1. Schulz B, Boyle C, Draeger S and Rommert AK. Endophytic fungi: a source of novel biologically active secondary metabolites. Mycological Research. 106, 2002, 996-1004.
2. Strobel G and Daisy B. Bioprospecting for microbial endophytes and their natural products. Microbiol. Mol. Biol. Rev. 67(4), 2003, 491-502.
3. Tan RX and Zou WZ. Endophytes: a rich source of functional metabolites. Nat. Prod. Rep. 18, 2001, 448-459.
4. Strobel G, Daisy B, Castillo U, Harper J. Natural products from endophytic microorganisms. Journal of Natural Products. 67, 2004, 257-268.
5. Singh HB, Singh RS, Shandhu JS. Herbal Medicine of Manipur, Daya Publishing House, Delhi, India. 2003.
6. Schulz B, Wanke U, Draeger S, Aust HJ. Endophytes from herbaceous plants and shrubs effectiveness of surface sterilization methods. Mycological research. 97, 1993,1447-1450.
7. Suryanarayanan TS, Murali TS, Venkatesan G. Endophytic fungal communities in leaves of tropical forest trees: diversity and distribution patterns. Current Science. 85, 2003, 489-493.
8. Huang WY, Cai YZ, Hyde KD, Corke H and Sun M. Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. Fungal Diversity. 33, 2008, 61-75.
9. Ellis MB. Dematiaceous Hyphomycetes. The Cambrian News Ltd., Aberystwyth. Commonwealth Mycological Institute Kew, Surrey, England. 1976.
10. Watanbe T. Pictorial Atlas of Soil and Seed Fungi Morphologies of Cultured Fungi and Key to Species. Second Edition. CRC Press. 2002.
11. Barnett HL, Hunter BB. Illustrated Genera of Imperfect Fungi. APS Press, St. Paul, Minnesota, USA. 1998.

12. Oldenburg KR, Vo KT, Ruhland B, Schatz PJ, Yuan Z. A dual culture assay for detection of antimicrobial activity. J Biomol Screen. 1, 1996, 123–130.
13. Warjeet SL, Brajeshwari DK. Studies on the chemical constituents and antioxidant activities of the extracts from the roots of *Smilax lancaefolia* Roxb., Natural Product Res. 24(12), 2010, 1168-1176.
14. Kharwar RN, Verma VC, Strobel G, Ezra D . The endophytic fungal complex of Catharanthus roseus (L.) G. Don. Curr Sci India 95(2), 2008, 228–233.
15. Bhagobaty RK, Joshi SR, Kumar R. Penicillium verruculosum RS7PF: a root fungal endophyte associated with an ethno medicinal plant of the indigenous tribes of Eastern India. Afr J Microbiol Res. 4(9), 2010, 766–770.
16. Lacap DC, Hyde KD, and Liew ECY. An evaluation of the fungal 'morphotype' concept based on ribosomal DNA sequences. Fungal Diversity, 12, 2003, 53–66.
17. Xu LJ, Zhou LG, Zhao JL and Jiang WB. Recent studies on the antimicrobial compounds produced by plant endophytic fungi. Nat. Prod. Res. Dev. 20, 2008, 731-740.
18. Huang WY, Cai YZ, Corke H and Sun M. A Potential antioxidant resource: Endophytic fungi from medicinal plants. Economic Botany. 61(1), 2007, 14-30.

Source of Support: Nil, **Conflict of Interest:** None.