

RESEARCH ARTICLE

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Antimicrobial Activity of Endophytic Fungi Isolated from *Eryngium foetidum*, an Ethnomedicinal Plant of Assam

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ABSTRACT

Eyngium foetidum L. is a medicinal plant widely used by ethnic tribal communities of Assam as an alternative source of medicine for the treatment of various diseases. The present investigation was undertaken with an aim to isolate, identify and assess the antimicrobial activity of endophytic fungi associated with the healthy leaf tissues of *E. foetidum*. The endophytic fungi were isolated three different media, namely, Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Water Agar (WA) media and from three different sites. Altogether, 84 endophytic fungal isolates were isolated from 150 segments of leaf tissues. Dominant endophytes were found to be fungi belonging to the genus *Collectorichum*, followed by non-sporulating members grouped under mycelia sterilia. Other fungal genera that were isolated as endophytes were *Scopulariopsis, Cladosporium, Stemphylium, Penicillium* and *Alternaria*. The endophytic fungi thus isolated were studied for antimicrobial activity against some clinically significant human pathogenic test organisms. Ethyl acetate extracts of all endophytes exhibited antimicrobial activity by inhibiting at least one out of all the test pathogens. Amongst the isolated fungi, extracts obtained from three endophytes showed wide-spectrum activity against all the test organisms. The fungal endophytes were identified as *Scopulariopsis* sp., *Penicillium* sp. and a sterile isolate morphotype strain EF6. The study indicated that *E. foetidum* harbours a wide range of endophytes with antimicrobial properties and further detailed investigation of the compound present in them would lead to their potential therapeutic applications as a new source of medicine.

Keywords: Medicinal plant, *Eryngium foetidum* L., Endophytic fungi, Antimicrobial activity.

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INTRODUCTION

Medicinal plants have been used as potential sources of drugs since thousands of years both folk medicines as well as development of new modern and commercial medicines. In the recent times, these plants have been overexploited for obtaining plant-derived drugs in commercial levels. ^[1] This commercialization demands higher amounts of raw plant biomass for production of even a small amount of potentially active drug. In addition to this, increased reports of developing resistance by most of the pathogenic fungi and bacteria against already available commercial drugs have become a serious cause of concern for the health services around the world. ^[2] As such, a thorough search for new and effectual antimicrobial agents is indispensable and this can only be done by exploring new niches and habitats. ^[3-4]

It has been found that all plants harbour a wide range of non-pathogenic microflora within their tissues which are known as endophytes. [5] Endophytic organisms, particularly, fungi residing in the medicinal plants are capable of biosynthesizing pharmacologically active secondary metabolites similar to those produced by the host plants. [6] These endophytic microorganisms are renowned producers of bioactive secondary metabolites like terpenoids, lactones, steroids. quinones, alkaloids, isocoumarins, phenylpropanoids, and phenols amongst many others. [7] Plant species used in traditional medicine has been reported to play a significant role for exploration of new bioactive strains of endophytic fungi. Another possibility might be that their beneficial qualities are a result of the metabolites produced by their endophytic microbes. [8-9]

Eryngium foetidum L. is a medicinal herb widely used alternative to modern medicine for treatment of a number of ailments. The leaves of this plant have been recognized to have anti-diarrhoeic, anti-helmenthic, anti-inflammatory, anti-convulsant, anti-microbial and anti-malarial properties and as well as found to be effective against snakebites, as wound-healers and also against infertility complications. It has been reported to be used against constipation, asthma and stomachache. ^[10-11] There are quite few reports on endophytic fungi of E. foetidum one of them being from Western Ghats and Biligirirangana hill of southern India. [12-13] There is also report of novel bioactive compound Xylaropyrone obtained from the endophytic fungus, Xylaria feejensis isolated from E. foetidum of Thailand. [14] Such results exemplify that E. foetidum are colonized by endophytic fungi that can produce bioactive metabolites. Strobel and Daisy (2003) were of the opinion that areas with high biodiversity as well as high numbers of endemic plant species might be the most possible niches for endophytes with novel chemistry. ^[15] Assam, which is amongst the states of North east India, is rich in medicinal plants. Eryngium foetidum L. is one such plant which is widely used by the ethnic tribal communities of Assam for treatment of various ailments. Therefore the present study was directed to isolate and identify endophytic fungi associated with healthy leaf tissues of E. foetidum with an aim to screen the isolates for antimicrobial activities against some clinically significant human test pathogens so that potent isolate could be studied further antimicrobial agents.

MATERIALS AND METHODS Sample collection

Healthy plant samples of *E. foetidum* were collected from three different sites of Assam, namely, Amingaon(26°11'5" N and 91°40'9" E), BaihataChariali (26°20'41"N and 91°43'35"E) and Sonapur (26°06'57.5"N and 91°58'44.8"E). From each site leaves of five individual healthy plants were selected randomly and collected in sterile polyethene bags. The collected plant species was identified by taxonomist via leaf and flower morphology and preserved in the Gauhati University Herbarium (accession no. 18539), Department of Botany. The samples were immediately brought to the laboratory and processed for isolation of endophytic fungi.

Isolation of endophytic fungi

Healthy leaves of E. foetidum were surface sterilized following Sushma et al. (2018) with slight modifications. They were sequentially dipped in 70% ethanol (2 min), followed by 0.5% sodium hypochlorite (1 min) then rinsed twice thoroughly with sterile distilled water (1-2 min). The leaves were then allowed to surface dry under sterile conditions. Small circular fragments of leaves were punched out measuring 0.5 mm in diameter with the help of a sterile puncture. The surface sterilized fragments were then placed in three different mycological media namely, Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Water Agar (WA) media supplemented with streptomycin sulphate $(50\mu g/ml)$ and incubated at 25 ± 2°C for 2 weeks. Fragments plated were observed once a day for the growth of endophytic fungi. Hyphal tips of fungi growing out of the surfaced sterilized leaf tissues were immediately transferred onto PDA slants and stored at 4°C for further study. The effectiveness of surface sterilization procedure was tested according to the method described by Schultz et al. (1998) by rubbing a surface sterilized leaf on a sterile PDA plate. Absence of any contaminant or fungal growth proved the efficacy of the protocol used. [16]

Identification of endophytic fungal isolates

The fungal isolates were identified based on their morphological and microscopically reproductive characters observed when grownon PDA using standard identification manuals. ^[17-19] The fungal isolates that failed to sporulate were categorized as *Mycelia sterilia*. Endophytic fungal isolates under *Mycelia sterilia* with distinct morphological features were designated as Morphotype.

Fungal diversity data analysis

The relative colonization frequency (CF%) of endophytic species was calculated using the formula:

$$CF \% = (N_{col} / N_t) \times 100$$

Where, N_{col} stands for the number of segments colonized by each endophytic fungal species, and N_t stands for the total number of segments plated on media.

Dominant endophytic fungi recovered was calculated as percentage colony frequency divided by sum of percentage of colony frequency of all endophytes X 100. [20-21]

Fungal cultivation and metabolites extraction

Actively growing pure endophytic fungal isolates were cultivated in Potato Dextrose Broth (PDB) in 250 ml Erlenmeyer flasks containing 100 ml of the medium each. The fungal isolates were grown in BOD shaking incubator at 28°C for about 2 weeks with a periodic

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shaking of 120 rpm. Fungal culture was then filtered out through sterile whatman filter paper to remove the mycelial mats. The liquid broth was then collected and extracted with 100ml of ethyl acetate (EtOAc) each using a separating funnel after vigorously shaken for 10-15 min. Cell mass was discarded and the solvent so obtained was allowed to evaporate off using a rotary evaporator. Evaporation of the ethyl acetate yielded the crude extracts which was then dissolved in dimethyl sulphoxide (DMSO) and stored at 4°C for determination of antimicrobial activity.

Determination of antimicrobial activity

Antimicrobial activity of the crude metabolites was determined by agar cup diffusion method against some clinically significant human test pathogens. The test pathogens include one gram positive bacterium: Staphylococcus aureus (MTCC 737); two gram negative Pseudomonas aeruginosa bacteria: (MTCC 424), Escherichia coli(MTCC 443) and one pathogenic fungus Candida albicans (MTCC 227) all of which were procured from the Institute of Microbial Technology (IMTECH), Chandigarh, India. The bacterial test pathogens were cultivated on freshly prepared Nutrient Broth while the fungal test pathogen was cultured on Sabouraud's Dextrose Broth. For bacterial pathogens, nutrient agar plates were inoculated with 0.2 ml of overnight grown culture containing 1.0×106 cells. Similarly, for fungal pathogen, Sabouraud's dextrose agar plate was inoculated with 0.2 ml of culture containing 1.0×10⁶ cells. The test organisms were evenly spread out on respective plates using a sterile cotton swab and agar cups were prepared on them using a sterile cork borer (7 mm in diameter). The agar cups were then filled in with 100µL of the culture filtrate of each endophytic fungus and incubated at 37±1°C for 24 hours for bacterial and at 28 ± 1°C for 48 hours for fungal pathogens. The antimicrobial activity of the extracts was determined by appearance of clear zone of inhibition against the target organism around the agar cups. DMSO was used as the negative control whereas Streptomycin (10µg) and Fluconazole (25µg) were used as positive control.



Fig. 1: Endophytic fungi growing out from surface sterilized leaf fragment of *E. foetidum*

RESULTS Isolation and identification of endophytic fungi from *E. foetidum*

In the present study, endophytic fungi were isolated from healthy, symptomless leaves of *E. foetidum* from three different sites and in different media. Out of the 150 leaf segments plated, a total number of 84 endophytic fungal isolates were recovered belonging to species under different genera. The highest recovery of endophytes was obtained in WA medium (28.67%), followed by MEA (16.67%) and the least was in PDA medium (10.67%) [Table 1].

 Table 1: Endophytes recovered from *E. foetidum* leaf on different media from various sites.

Sample	Media	No. of colonies recovered per location			Total no. of fungal colonies	% of Recovery
		AN	BH	SN	recovered/150 segments	Recovery
Leaf	PDA	7	5	12	16	10.67
	MEA	5	9	14	25	16.67
	WA	4	9	17	43	28.67
	Total isolates	16	25	43	84	56.01

PDA= Potato Dextrose Agar; MEA=Malt Extract Agar; WA=Water; AN=Amingaon, BH=Baihata, SN=Sonapur.

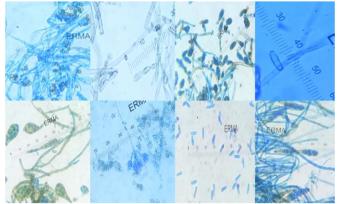


Fig. 2: Microscopic photographs of some isolated endophytic fungia) Scopulariopsis sp., b)Cladosporium macrocarpum, c) Alternaria sp., d) Cladosporium fulvum, e) Stemphylium sp. 1, f) Penicillium sp., g) Colletotrichum sp., h) Stemphylium sp. 2 (under 40x)

The total colonizing frequency (CF%) of endophytic fungi in healthy leaf tissues of E. foetidum was found to be 56% out of which the genus Colletotrichum showed the highest colonization frequency (32.67%), followed by the non-sporulating isolates categories as morphotypes (15.33%). The other endophytic fungal isolates were Scopulariopsis sp. (0.67%), Cladosporium macrocarpum, Cladosporium fulvum (0.67%), Stemphylium sp. (4%), Penicillium sp. (0.67%) and Alternaria sp. (0.67%) [Table 2]. The commonly isolated species from all the sampling sites was found to befungi belonging to genera Colletotrichum. Amongst the genera of Colletotrichum, the most dominant isolate was found to Colletotrichum sp.10 with a colonization frequency of 11.33%. The non-sporulation fungal groups which were designated as morphotypes did not produce any sexual or asexual propagules both on synthetic or host extract amended media. Among them, morphotype sp.1 showed the highest colonization frequency of 9.33%. Maximum number of isolates were recovered from Sonapur (43), followed by Jalukbari (25) and the least was from Amingaon (16).

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Endophytic fungi	AN	BH	SN	Total isolates/150 fragments	CF (%)	Frequency of dominant endophytes (%)	
Scopulariopsis sp.	1			1	0.67	1.19	
Cladosporium macrocarpum	4			4	2.67	4.76	
Cladosporium fulvum	1			1	0.67	1.19	
Stemphylium sp.1		4	1	5	3.33	5.94	
Stemphyliums p.2		1		1	0.67	1.19	
Penicillium sp.		1		1	0.67	1.19	
Alternaria sp.1		1		1	0.67	1.19	
Alternaria sp.2		1		1	0.67	1.19	
Colletotrichum sp.1		1		1	0.67	1.19	
Colletotrichum sp.2		1		1	0.67	1.19	
Colletotrichum sp.3		1		1	0.67	1.19	
Colletotrichum sp.4		1		1	0.67	1.19	
Colletotrichum sp.5		1		1	0.67	1.19	
Colletotrichum sp.6		4		4	2.67	4.76	
Colletotrichum sp.7		2		2	1.33	2.37	
Colletotrichum sp.8			2	2	1.33	2.37	
Colletotrichum sp.9			8	8	5.33	9.52	
Colletotrichum sp.10			17	17	11.33	20.23	
Colletotrichum sp.11			5	5	3.33	5.94	
Colletotrichum sp.12			5	5	3.33	5.94	
Morphotype sp.1	10	4		14	9.33	16.66	
Morphotype sp.2		2		2	1.33	2.37	
Morphotype sp.3			2	2	1.33	2.37	
Morphotype sp.4			1	1	0.67	1.19	
Morphotype sp.5			1	1	0.67	1.19	
Morphotype sp.6			1	1	0.67	1.19	
Total no. of isolates	16	25	43	84	56.01		

Table 2: Composition of endophytic fungi in healthy leaf tissues of *E. foetidum* isolated from three different sites of Assam.

*CF=Colonization Frequency; AN= Amingaon, BH= Baihata , SN=Sonapur

Isolate code	Endophytic fungi	Candida albicans	Staphylococcus aureus	Escherichia coli	Pseudomonas aeruginosa
EF1	Scopulariopsis sp.	++	++	++	++
EF2	Cladosporium macrocarpum	++	+	+	+
EF12	Cladosporium fulvum	++	+	++	+
EFB4	Stemphylium sp.1	+++	+	+	+
EFB5	Stemphylium sp.2	++	++	+	+
EFB9	Penicillium sp.	+++	+++	++	+++
EFB16	Alternaria sp.1	++	+	+	+
EFB17	Alternaria sp.2	++	+	+	+
EFB1	Colletotrichum sp.1	+	+	+	+
EFB2	Colletotrichum sp.2	++	+	+	+
EFB3	Colletotrichum sp.3	++	+	+	+
EFB14	Colletotrichum sp.4	++	+	+	+
EFB15	Colletotrichum sp.5	++	+	++	+
EFB18	Colletotrichum sp.6	++	+	++	+
EFB22	Colletotrichum sp.7	++	+	+	+
EFS1	Colletotrichum sp.8	++	+	+	++
EFS2	Colletotrichum sp.9	++	+	+	+
EFS4	Colletotrichum sp.10	++	+	++	+
EFS14	Colletotrichum sp.11	+		+	+
EFS28	Colletotrichum sp.12	++		+	+
EF6	Morphotype sp.1	++	+++	++	++
EFB6	Morphotype sp.2	++		+	+
EFS13	Morphotype sp.3	+	+	+	
EFS15	Morphotype sp.4	+	+		+
EFS35	Morphotype sp.5	+	++		+
EFS41	Morphotype sp.6	+		+	

"--"no inhibition; "+"> 10 mm; "++"> 15 mm; "+++" >20 mm.

Antimicrobial activity of endophytic fungi

The isolates were screened for antimicrobial activity against some clinically important pathogenic human test organisms. The result showed that all of the fungal isolates displayed antimicrobial activity by inhibiting at least one of the test pathogens (Table 3). It was also observed that most of the isolates were effective in inhibiting all the test pathogens considered. However, comparatively the isolates showed higher antifungal activity against *Candida albicans* than the bacterial pathogens. Amongst the isolates three endophytic fungi showed considerable antimicrobial activity by

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inhibiting three bacterial pathogens and one pathogenic fungus. The isolates were identified as *Scopulariopsis* sp., *Penicillium* sp. and non-sporulating isolate morphotype sp.1 (strain EF6). The ethyl acetate extracts of these three fungi showed very effective inhibition against all the four test pathogens by agar diffusion assay (Fig. 3 & 4).

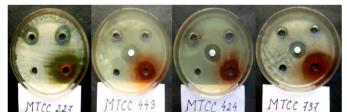


Fig. 3: Antimicrobial activity of ethyl acetate extract of *Penicillium* sp. against test pathogens



Fig. 4: Antimicrobial activity of ethyl acetate extract of *Scopulariopsis* sp. and non-sporulating isolate, Morphotype sp.1 against test pathogens

DISCUSSION

Endophytic fungi are ubiquitous in nature and known to be distributed naturally in both temperate and tropical regions. Reports suggest the presence of numerous endophytic fungi within medicinal plants that are involved in the co-production of active metabolites. [22] In the present study, endophytic fungi assemblages of *E. foetidum*, an ethno-medicinal plant of Assam in North-East India was investigated for antimicrobial metabolites. A total of 84 endophytic fungal strains were isolated from surface sterilized leaf fragments of *E. foetidum* on three different mycological media (PDA, MEA and WA). The highest percentage recovery of endophytes from the leaves of this was observed from WA media (28.67%). Similar recovery of endophytes from E. foetidum was also observed from WA media in earlier works where isolation of endophytes was done from various parts of the plant from the Western Ghats of India. [12] However there are no reports of endophytes being isolated from the leaves of this plant, which is the mostly used part in ethnic pharmacology in the North East India. Amongst the sites, Sonapur an unpolluted area harboured maximum endophytes while least was obtained from Amingaon which is an area with rapid industrial development. The decrease in the abundance and diversity of fungal communities might be the effect of toxic metals air pollutants as also reported in Pine needles. [23] The endophytes consisted of fungi belonging to different genera Colletotrichum, Scopulariopsis, Cladosporium, Stemphylium, Penicillium, Alternaria and non-sporulating fungi categorized as mycelia sterilia and isolates with distinct cultural morphology were conventionally classified as morphotypes. Alternaria was found to be isolated as endophytic fungi from E. foetidum in Biligirirangana hill, India.^[13] The result showed that the genus Colletotrichum was isolated from two of the three study sites while non-sporulating fungal genera were mostly isolated from Jalukbari site. In various instances, the genus Colletotrichumhas been reported as dominant endophytic fungi from several medicinal plants species. ^[24-26] However, this is the first report from E. foetidum leaf tissues. The variation of endophytic colonization frequency among the sites might due to various environmental factors. Many workers were of the opinion that endophytic fungal communities changes through time and space and is influenced by climatic and environmental conditions. [27-28]

Another important aspect of studies on endophytic microbes, especially, fungi is their latent ability to produce a large array of bioactive compounds that can defend the plant against various pathogenic organisms. ^[15, 29] For instance, natural compounds synthesized by endophytic fungi have been reported to have inhibitory effect against varied types of animal and plant pathogens. [30-31] Similar was the case in the typical example of taxol, an anticancer agent produced by Taxus brevifolia and its associated endophyte, Taxomyces andreanae. [32] Reports are found to propose that the medicinal properties of a plant might be the result of the capacity of its associated endophytes to produce biologically active secondary metabolites. ^[33] As such, the isolation and identification of endophytic mycobiota is necessary to have an insight to their bioactive potential. In the present study, a total of 25 strains out of 84 (29.76%) showed antimicrobial activity most of which (18 out of 25) showed wide spectrum activity. The three most potent isolates that effectively inhibited the test pathogens were Scopulariopsis sp. (strain EF1), Penicillium sp. (strain EFB9) and morphotype sp.1 (strain EF6). However, amongst the isolates, ethyl acetate extract obtained from Penicillium extract was found to be most effective in inhibiting all the four test pathogens. Reports suggest that various parameters like difference in incubation temperature, changes in composition of media and degree of aeration influence the amount and type of compound produced by an endophytic fungus. ^[34] In addition, these parameters might either increase or decrease the production of bioactive compounds by the particular endophytic fungi. For this reason, further tests are needed to evaluate the biological activity of the strains that showed inhibition during the fermentation assay. Moreover, our interpretation clearly specifies that endophytic fungi from leaves of E. foetidum have pharmaceutical potential as they might produce antimicrobial compounds. Also, the therapeutic properties of this plant might be an outcome of the capacity of endophytic microorganisms to produce biologically active secondary metabolites. Advanced studies into this area is now essential to identify the active compounds produced in order to discover novel drugs with antimicrobial activity.

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