



## RESEARCH ARTICLE

# Endophytic fungal assemblages of *Zanthoxylum oxyphyllum* Edgew. and their antimicrobial potential

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## ABSTRACT

*Zanthoxylum oxyphyllum* Edgew. is a medicinal plant widely been used by the local tribal communities of Assam as an alternative source of medicine for the treatment of various diseases. In the present study, endophytic fungi associated with *Z. oxyphyllum* were undertaken with an aim to investigate the isolates for their antimicrobial potential. The endophytic fungi were recovered using four different media, namely, Malt Extract Agar (MEA) media, Potato Dextrose Agar (PDA) media, Water Agar (WA) media and media amended with the Plant Extract (PEA) from samples collected from three sites. Altogether, 18 isolates of endophytic fungi were isolated from 150 surface sterilized and healthy leaf fragments. *Colletotrichum* was found to be dominant endophytic genus with 7 different species. Other isolated endophytic fungal genera were *Fusarium*, *Curvularia*, *Aspergillus*, *Corynespora* and isolates belonging non-sporulating fungi categorised as Mycelia Sterilia. The endophytic fungi were determined for antimicrobial activity against selected clinically significant human pathogenic test organisms. Ethyl acetate crude extracts of all endophytic fungi exhibited antimicrobial activity by inhibiting a minimum of one of the four test pathogens. Amongst the isolates, crude extracts obtained from *Fusarium* sp. and five *Colletotrichum* spp. showed wide-spectrum antimicrobial activity against all the test organisms. The study indicated that *Z. oxyphyllum* harbours a wide range of endophytes capable of producing secondary metabolites with antimicrobial properties. Further detailed investigation of their bioactive metabolites might lead to discovery of compounds with potential therapeutic applications as a new source of medicine.

## Introduction

Plants known to possess medicinal values have been used in traditional folk medicine by ethnic tribal communities all over the world including India. They are also potential sources of drugs since long back and recently used for the development of modern and commercial medicines. Currently, medicinal plants used in the commercial drug industry have been overexploited for the production of plant-derived drugs. This has led to an increase in the amount of plant biomass needed for the production of even a small quantity of potentially active commercial drugs. Additionally, there are increased reports of resistance developed by most of the pathogenic microorganisms against already available commercial drugs (1). This has become a serious concern for the health services around the world. Therefore, a thorough search for

new and effective antimicrobial agents is indispensable and this can only be done by exploring new niches and habitats (2, 3). Plants all over the globe are reported to harbor a wide range of non-pathogenic microflora within their tissues which are known as endophytes (4). Endophytic microorganisms, especially fungi inhabiting medicinal plants have the ability of synthesizing bioactive secondary metabolites analogous to those produced by their respective host plants (5). These microorganisms are prominent producers of bioactive secondary metabolites like terpenoids, lactones, steroids, quinones, alkaloids, isocoumarins, phenyl propanoids and phenols (6). Plants growing in regions with high biodiversity having medicinal properties and used in traditional medicine has been reported to be a significant area for exploration of new bioactive strains of endophytic

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fungi. Researchers 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 (7). Another prospect also suggests that the valuable medicinal qualities of such plants might be a consequence of the metabolites produced by their endophytic microflora (8).

*Zanthoxylum oxyphyllum* Edgew. belonging to the family Rutaceae is a spiny shrub that sometimes adapts to climbing habit. It is commonly known as Prickly ash and by various other local names in Assam like Mezenga or Mejenga (Assamese) and Onger (by Mising tribe of Assam). The plant has been used as traditional medicine by the ethnic communities of the North East India, especially by the tribal population of Assam. Tender shoots and leaves of this plant are taken as vegetable and also in non-vegetarian dishes. They have been found to aid against stomach disorder, act as a blood purifier as well as against leucoderma (9). Fruits of *Z. oxyphyllum* are used as spice and are known to help in digestive disorders. The bark of the plant is commonly applied to treat skin diseases, rheumatism, ulcers, varicose veins, leg aches, inflammations, fever and hypotension. In addition to this, it also has stimulant, astringent and digestive properties and is used for the treatment of dyspepsia and diarrhoea (10). Studies show that endophytic fungi inhabiting medicinal plants synthesize natural compounds that possess an inhibitory effect against pathogenic microorganisms as well as cure various diseases (11). The typical example is that of the first compound "taxol", an anticancer agent that is produced by *Taxus brevifolia* and its associated endophyte, *Taxomyces andreanae* (12). The isolation and identification of endophytic mycobiota are thus necessary to have an insight to their bioactive potential. However, there are no reports on the endophytic fungi of this plant species in spite of their wide medicinal uses. *Z. oxyphyllum* is one such plant which is widely used by the ethnic tribal communities of Assam for the treatment of various ailments. Therefore, the present study was directed to isolate and identify endophytic fungi associated with healthy leaf tissues of *Z. oxyphyllum* with an aim to screen the isolates for antimicrobial activities against some selected clinically significant human test pathogens so that potent isolate could be studied further to discover potential antimicrobial agents.

## Materials and Methods

### Sample collection

Healthy plant samples of *Zanthoxylum oxyphyllum* were collected from three different locations of Assam, such as Jonai (27.8323° N, 95.2214° E), Boko (25.9778° N, 91.2356° E) and Goalpara (26.1641° N, 90.6252° E). Leaves of five individual plants from each site were randomly selected and collected in sterile polyethene bags. The collected plant specimen was identified by referring herbaria and authenticated by Dr. Souravjyoti Borah (Taxonomist). A voucher specimen is being deposited in the Gauhati University Herbarium, Department of Botany (GUBH)

with an accession number 18767. Plant samples were then brought to the laboratory for the isolation of endophytic fungi.

### Isolation of endophytic fungi

Since there are no earlier reports of isolation of endophytic fungi from *Zanthoxylum oxyphyllum*, healthy leaf tissues were surface sterilized by developing a standard protocol. Leaves were sequentially dipped in 70% ethanol (2 min), followed by 0.5% sodium hypochlorite (1 min) and then rinsed twice with sterile distilled water (1 min each time). Leaves were then allowed to surface dry under a Laminar Air Flow Chamber. Small circular fragments of leaves measuring 0.5 mm in diameter were punched out with the help of a sterile puncture. These sterilized leaf fragments were then placed in four different mycological media namely, Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), Water Agar (WA) media and also media amended with the Plant Extract (PEA) that were supplemented with streptomycin sulphate (50 µg/ml) and incubated at 25±2°C for about 2 weeks. The effectiveness of surface sterilization process was verified according to the standard method of rubbing a surface sterilized leaf on a sterile PDA plate (13). Fragments plated were observed daily for the growth of endophytic fungi. Fungal hyphae found growing from the surfaced sterilized leaf fragments were immediately transferred onto PDA slants and stored at 4 °C for further study. Absence of any contaminant or fungal growth proved the efficacy of the protocol used.

### Identification of endophytic fungal isolates

Fungal isolates thus recovered were identified on the basis of their morphological and reproductive characters observed microscopically, grown on PDA using standard identification manuals (14-16). The fungal isolates that failed to sporulate were categorized as mycelia sterilia and those having distinct morphological features were designated as morphotype. The isolated endophytic fungi were coded according to the site of collection as Zoj (*Z. oxyphyllum* from Jonai), ZOB (*Z. oxyphyllum* from Boko) and ZOG (*Z. oxyphyllum* from Goalpara).

### Fungal diversity data analysis

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 (17).

Dominant endophytic fungi recovered was calculated as percentage colony frequency divided by sum of percentage of colony frequency of all endophytes X 100 (18).

### Extraction of metabolites

Pure endophytic fungal isolates were cultivated in Potato Dextrose Broth (PDB) in Erlenmeyer flasks containing 100 ml of the medium each. Further, it was incubated in a BOD shaking incubator (Wosico WSW-132) at 28 °C for 2 weeks with a periodic

shaking at 120 rpm. The fungal mycelia were filtered out through sterile Whatmann filter paper. Each of the liquid broth was then extracted with equal amount of ethyl acetate (EtOAc) using a separating funnel after vigorous shaking for 10-15 min. The supernatant was allowed to evaporate using a rotary evaporator (Wosico WSW-191) and the crude extracts were obtained. The crude extracts were then dissolved in 100% dimethyl sulphoxide (DMSO) and stored at 4 °C for determination of antimicrobial activity (19).

### Determination of antimicrobial activity

Antimicrobial activities of the extracts were determined through agar well diffusion method (20, 21) against selected clinically important human pathogens. These included one gram +ve bacterium *Staphylococcus aureus* Rosenbach (MTCC 737); two gram -ve bacteria *Pseudomonas aeruginosa* (Schroeter) Migula (MTCC 424) and *Escherichia coli* (Migula) Castellani & Chalmers (MTCC 443); and a fungal strain *Candida albicans* (Robin) Berkhout var. *albicans* (MTCC 227). The reference strains were procured from the Institute of Microbial Technology (IMTECH), Chandigarh, India. All the selected bacterial test pathogens as well as the fungal test pathogen were cultivated in liquid culture media i.e., on Nutrient Broth and Sabouraud Dextrose Broth respectively. Nutrient agar plates were then inoculated with 0.2 ml of bacterial culture containing  $1.0 \times 10^6$  cells. Likewise, Sabouraud Dextrose Agar plate was inoculated with 0.2 ml of fungal culture containing  $1.0 \times 10^6$  cells. They were evenly spread over 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 filled with 100 µl of crude extract of each endophytic fungus and incubated at  $37 \pm 1$  °C for 24 hrs and at  $28 \pm 1$  °C for 48 hrs for bacterial and fungal pathogens respectively. The antimicrobial activity of the crude extracts was determined by measuring the clear zone of inhibition against the pathogenic organism around the agar cups. Dimethyl sulphoxide (DMSO) was used as negative control, whereas streptomycin sulphate (10 µg) and Fluconazole (25 µg) were used as positive control.

### Data analysis

To evaluate the inhibitory effect of the extracts of endophytic fungi against the test pathogens, Dimensionality-reduction method, Principle Component Analysis (PCA) was used. Antimicrobial activity of the ethyl acetate extracts of the potent fungal isolates were analyzed against the four selected human test pathogens, namely, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans*. Correlation matrix in multivariate option was used to generate PCA biplots using PAST 4.02 PCA software (22, 23).

## Results

### Isolation and identification of endophytic fungi from *Zanthoxylum oxyphyllum*

Endophytic fungi were isolated from healthy leaves of *Zanthoxylum oxyphyllum* from three different sites of Assam in different media. Out of 150 leaf segments

plated, a total number of 74 endophytic fungal isolates were recovered. The highest recovery of endophytes was obtained in PDA media (21.33%), followed by PEA (10%), MEA (9.33%) and the least were in WA media (8.67%). Maximum number of isolates were recovered from Boko (36), followed by Goalpara (25) and the least was from Jonai (13) (Table 1). The total colonizing frequency (CF%) of endophytic fungi in healthy leaf tissues of *Z. oxyphyllum* was found to be 49.33% out of which isolates belonging to the genus *Colletotrichum* showed the highest colonization frequency (25.34%), followed by non-sporulating isolates categorised as morphotypes of *Mycelia sterilia* (12.66%) and the genus *Curvularia* (6.67%) (Table 2). Other isolates include *Fusarium oxysporum* (2%), *Corynespora cassicola* (2.70%) and *Aspergillus flavus* (1.33%) (Table 2) (Fig. 1). The commonly isolated species from all the sampling sites was found to be fungi belonging to the genera *Colletotrichum*. The second highest colonization frequency from all the sites was found to be that of non-sporulating fungi (*Mycelia sterilia*).

### Antimicrobial activity of endophytic fungi

Endophytic isolates were screened for antimicrobial activity against four clinically important pathogenic human test organisms. The result showed that all of the fungal isolates displayed antimicrobial efficacy by inhibiting at least one of the test pathogens (Table 3). It was also observed that most of the isolates inhibited all the bacterial test pathogens considered. Amongst the isolates, crude extracts of six species of *Colletotrichum* showed considerable antimicrobial activity by inhibiting all the three bacterial pathogens and the pathogenic fungus with high zones of inhibition ranging from 20-25 mm. Out of all the isolates, crude metabolites of *Colletotrichum gloeosporioides* showed the highest zones of inhibition against all the pathogens, followed by *C. acutatum* (Table 3). Metabolites of another isolate identified as *Fusarium oxysporum* showed very effective inhibition against all the four test pathogens (Fig. 2) (Table 3).

### Principle Component Analysis

PCA biplot analysis of the extracts reveals the correlation of endophytic fungi against the pathogenic test microorganisms in terms of zones of inhibition observed (Fig. 3). The two principal components explained 82.58% of the total variance. Component 1 explained 51.48% of the variance, while the Component 2 explained 31.10% of the variance. It was observed that fungal isolates belonging to the genus *Colletotrichum* except *C. dematium* and *C. acutatum* showed positive and higher antagonistic activity against *Staphylococcus aureus* and *Candida albicans*. Similar result was also observed for *Fusarium oxysporum*. However, *Colletotrichum siamense*, *C. gloeosporioides* and *C. boninense* showed positive correlation indicating better antibacterial activity against *S. aureus* and *Escherichia coli*. Similarly, *C. asianum*, *C. nymphaeae* and *F. oxysporum* also showed positive correlation indicating more promising antimicrobial activity against *C. albicans*. However, a negative correlation was observed in the case of *C. dematium* and *C. asianum* as it showed



**Table 1.** Endophytes recovered from *Z. oxyphyllum* leaf on different media from various sites.

Sample	Media*	No. of colonies recovered from sites			Total no. of fungal colonies recovered out of 150 segments	% of recovery
		JO	BO	GO		
Leaf	PDA	4	13	15	32	21.33
	MEA	2	9	3	14	9.33
	WA	1	7	5	13	8.67
	PEA	6	7	2	15	10.00
	Total isolates	13	36	25	74	

\*PDA= Potato Dextrose Agar; MEA=Malt Extract Agar; WA=Water Agar ; PEA= Plant Extract Agar; JO= Jonai, BO=Boko, GO=Goalpara.

**Table 2.** Endophytic fungal composition in healthy leaf tissues of *Z. oxyphyllum* isolated from three sampling sites of Assam.

Endophytic fungi	Locations			Total isolates/150 fragments	CF (%)	Frequency of dominant endophytes (%)
	JO	BO	GO			
<i>Fusarium oxysporum</i>	--	3	--	3	2.00	4.05
<i>Curvularia pallescens</i>	--	3	--	3	2.00	4.05
<i>Curvularia protuberata</i>	--	--	4	4	2.67	5.41
<i>Curvularia geniculata</i>	--	--	3	3	2.00	4.05
<i>Aspergillus flavus</i>	--	2	--	2	1.33	2.70
<i>Corynespora cassicola</i>	2	--	--	2	1.33	2.70
<i>Colletotrichum dematium</i>	5	--	--	5	3.33	6.75
<i>Colletotrichum siamense</i>	--	6	--	6	4.00	8.10
<i>Colletotrichum gloeosporioides</i>	--	7	--	7	4.67	9.47
<i>Colletotrichum acutatum</i>	--	4	--	4	2.67	5.41
<i>Colletotrichum boninense</i>	--	--	7	7	4.67	9.47
<i>Colletotrichum asianum</i>	--	--	4	4	2.67	5.41
<i>Colletotrichum nymphae</i>	--	--	5	5	3.33	6.75
Morphotype sp.1	3	--	--	3	2.00	4.05
Morphotype sp.2	3	--	--	3	2.00	4.05
Morphotype sp.3	--	5	--	5	3.33	6.75
Morphotype sp.4	--	6	--	6	4.00	8.10
Morphotype sp.5	--	--	2	2	1.33	2.70
<b>Total no. of isolates</b>	<b>13</b>	<b>36</b>	<b>25</b>	<b>74</b>	<b>49.33</b>	<b>100</b>

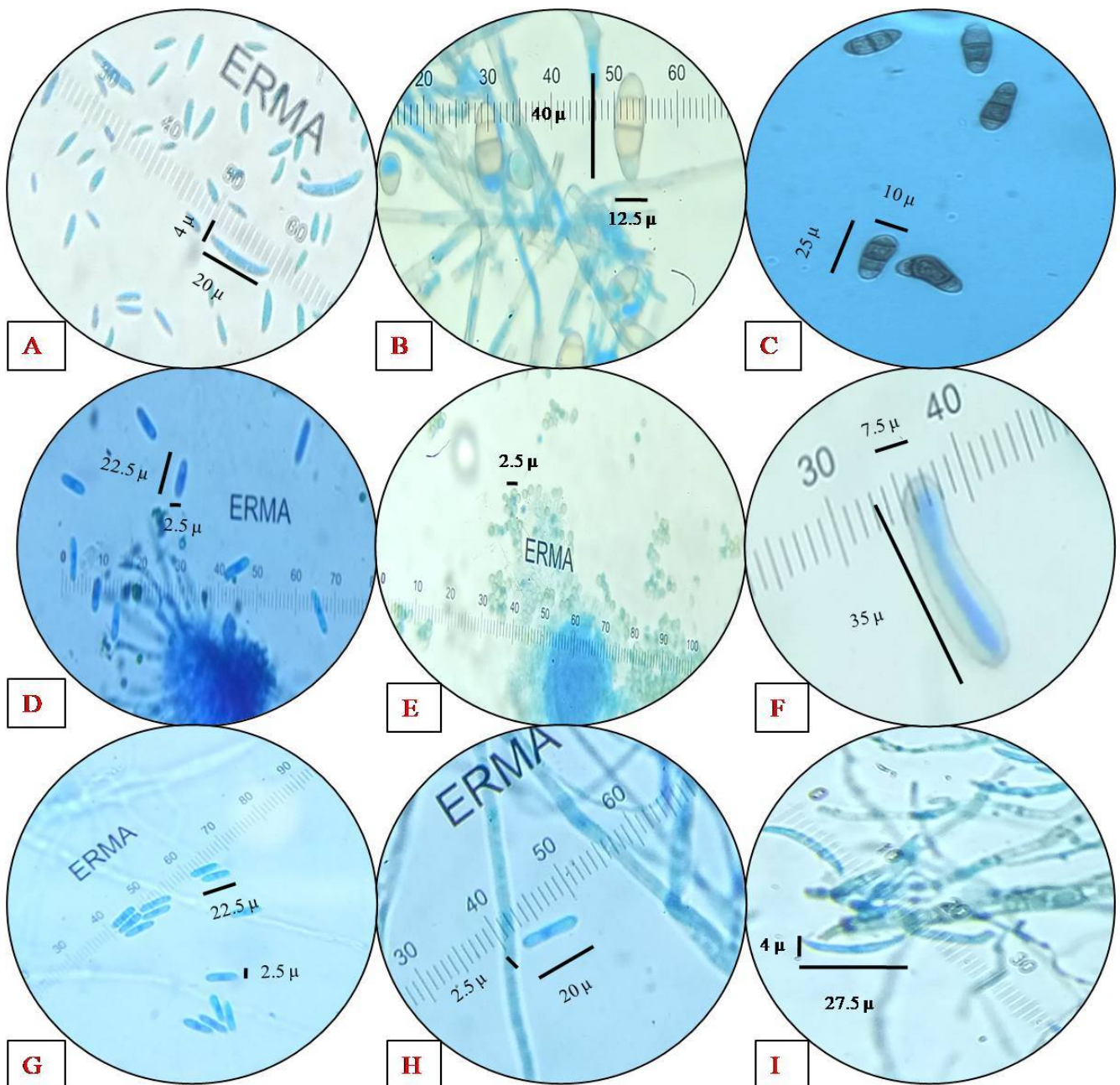
CF=Colonization Frequency; JO=Jonai, BO=Boko, GO=Goalpara

higher antimicrobial activity against *E. coli* in comparison to the other three test pathogens. The PCA analysis also suggests that the isolates belonging to the genus *Colletotrichum* and the isolate *F. oxysporum* showed similar trends of antagonistic activity against the test pathogens by showing more inhibitory effect against *S. aureus*, *C. albicans* and *E. coli* (Fig. 3).

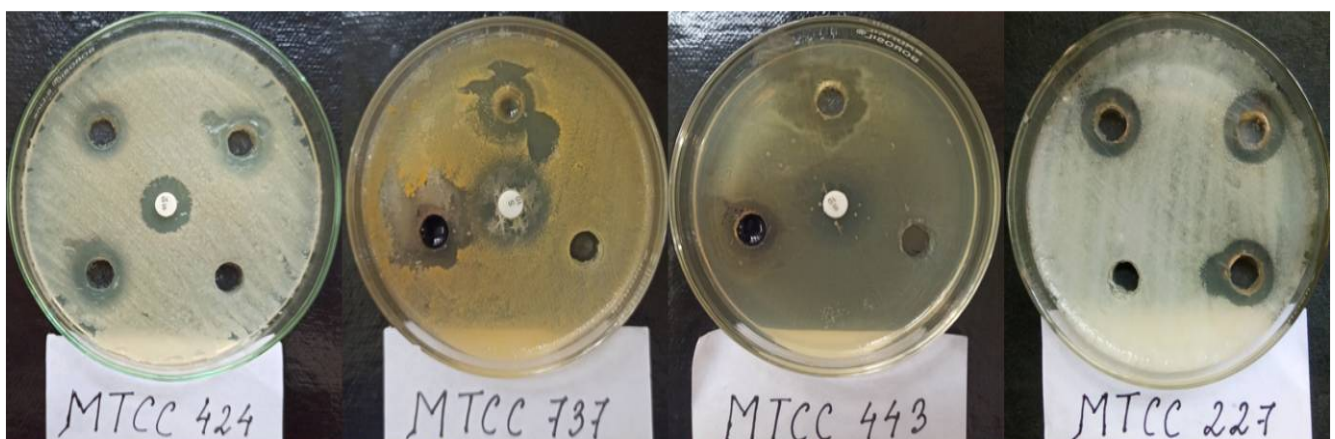
## Discussion

Endophytic fungi are found to be distributed naturally within plants of both temperate and tropical regions around the world. In the present study, endophytic fungi harbouring healthy leaf tissues of *Z. oxyphyllum*, an ethnomedicinal plant of Assam, was investigated for their antimicrobial potential as there were no earlier reports of endophytic fungal associated to this plant. The result clearly indicated the occurrence of endophytic fungal community within the leaves of *Z. oxyphyllum* and 74 isolates belonging to 18 different species of endophytic fungi were isolated from 150 surface sterilized leaf fragments on different media. The highest percentage recovery of endophytes from the leaves of this plant was observed in PDA media. However, recovery of endophytic fungi was found to

be merely similar in PEA and MEA media and the least recovery was observed from WA medium. Earlier reports on isolation of endophytic fungi have also indicated that PDA is the most suitable media for isolation of such fungi from medicinal plants and showed highest recovery of endophytes (24, 25). The maximum numbers of isolates were recovered from the samples collected from Boko and the least number of fungal colonies were isolated from the samples of Jonai. The variation and frequency of endophytic colonization among the sampling sites might be because of the environmental condition which corresponds with earlier reports on endophytic colonization (26). It has been reported that endophytic fungal communities vary according to time and space and is influenced by climatic and environmental conditions (27, 28). In this study, endophytic fungi belonging to different genera like *Colletotrichum*, *Fusarium*, *Curvularia*, *Corynespora*, *Aspergillus* and *Mycelia sterilia* were isolated from three different geographical sites of Assam. The genus *Colletotrichum* is isolated from all the three study sites considered for the study. This corresponds with earlier reports on colonization of endophytic fungi that suggest that the genus *Colletotrichum* is often isolated as dominant endophytic fungi from several medicinal plants species (29-31).



**Fig. 1.** Microscopic photographs of some isolated endophytic fungi- A) *Fusarium oxysporum*, B) *Curvularia pallescens*, C) *Curvularia geniculata*, D) *Colletotrichum gloeosporoides*, E) *Aspergillus flavus*, F) *Corynespora cassicola*, G) *Colletotrichum siamense*, H) *Colletotrichum asianum*, I) *Colletotrichum dematium*, (under 40x and 100x magnifications).

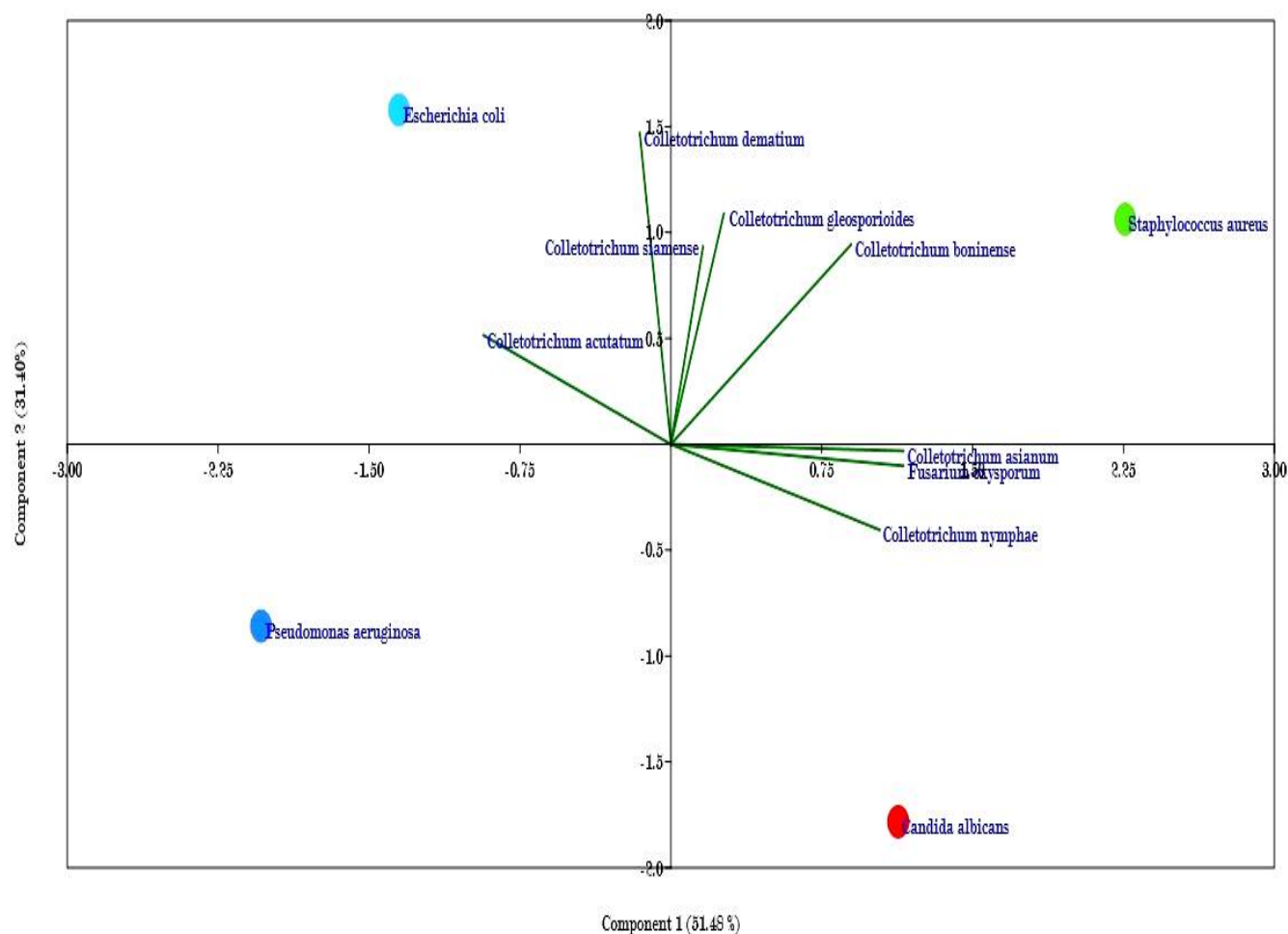


**Fig. 2.** Antimicrobial activity of the ethyl acetate extracts of some of the endophytic fungal isolates against selected test pathogens. (MTCC 424= *P. aeruginosa*; MTCC 737= *S. aureus*; MTCC 443= *E. coli*; MTCC 227= *C. albicans*).

**Table 3.** Antimicrobial activity of endophytic fungal isolates of *Z. oxyphyllum* against pathogenic test organisms.

Isolate code	Endophytic fungi	Zone of inhibition(mm ± SD)			
		<i>Candida albicans</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>
ZOB2	<i>Fusarium oxysporum</i>	23.8±0.57	23.5±1.00	17.6±1.52	17.0±1.00
ZOB4	<i>Curvularia pallescens</i>	12.3±1.52	11.3±1.52	11.6±0.57	13.6±0.57
ZOG3	<i>Curvularia protuberata</i>	17.1±1.25	12.1±1.04	16.0±1.00	11.1±0.76
ZOG4	<i>Curvularia geniculata</i>	16.3±0.57	0.00	12.5±1.80	0.00
ZOB14	<i>Aspergillus flavus</i>	11.6±0.57	13.6±0.57	10.3±1.52	11±1.00
ZOJ2	<i>Corynespora cassicola</i>	12.6±1.52	0.00	0.00	13.3±0.57
ZOJ1	<i>Colletotrichum dematium</i>	14.4±0.37	16.3±0.55	18.2±0.55	19.2±0.46
ZOB3	<i>Colletotrichum gloeosporioides</i>	24.4±0.47	24.7±0.60	24.1±0.56	23.8±1.04
ZOB9	<i>Colletotrichum siamense</i>	23.7±0.46	23.4±0.50	23.2±0.50	22.5±0.50
ZOB21	<i>Colletotrichum acutatum</i>	24.2±0.45	23.5±0.45	24.5±0.50	22.8±0.74
ZOG1	<i>Colletotrichum boninense</i>	24.1±0.25	24.4±1.00	23.0±0.45	18.9±0.90
ZOG8	<i>Colletotrichum asianum</i>	23.2±0.04	23.3±0.30	22.4±0.37	19.2±0.52
ZOG11	<i>Colletotrichum nymphae</i>	24.2±0.17	23.9±0.97	22.0±0.60	22.5±0.47
ZOJ3	Morphotype sp.1	12.0±1.00	11.3±1.52	17.6±0.57	0.00
ZOJ4	Morphotype sp.2	13.6±0.57	0.00	12.5±0.76	0.00
ZOB1	Morphotype sp.3	12.4±1.28	12.6±1.52	11.4±0.57	18.3±1.47
ZOB7	Morphotype sp.4	11.0±0.55	17.2±0.58	12.9±0.90	13.5±0.92
ZOG2	Morphotype sp.5	18.2±0.34	11.2±0.32	17.3±0.47	12.8±0.76

Values are mean of three replicates



**Fig. 3.** Analysis of principal component of potent endophytic fungal species against human test pathogens.

In the present study, the crude metabolites of 14 out of the 18 (77.78%) fungal strains showed prominent antimicrobial activity against at least one of all the test pathogens considered. The most potent

isolate that effectively inhibited all the test pathogens was *Colletotrichum gloeosporioides* (ZOB3). This was followed by the *C. siamense* (ZOB9), *C. boninense* (ZOG1), *C. acutatum* (ZOB21), *C. asianum* (ZOG8) and



*C. nymphae* (ZOG11). The crude extracts of the isolate identified as *Fusarium oxysporum* (ZOB2) also showed significant antimicrobial potential. However, all of the isolates showed significant antifungal activity. PCA analysis also clearly revealed the higher antimicrobial potential of the isolates belonging to the genus *Colletotrichum* as well as *F. oxysporum*. In many instances, endophytic fungi belonging to both of these genera have been reported to exhibit antimicrobial activity against a wide range of pathogens (32, 33). PCA biplot analysis also indicates a positive and higher efficiency of the isolates of genus *Colletotrichum* and *F. oxysporum* strain against mostly to *Staphylococcus aureus* and *Candida albicans*. Our results therefore clearly indicated that endophytic fungi obtained from leaves of *Z. oxyphyllum* have pharmaceutical potential as they produce antimicrobial compounds inhabiting the growth of pathogenic microorganisms. It can also be stated that the therapeutic properties of this plant could also be an outcome of endophytic colonization which produces biologically active compounds. Further evaluation of the bioactive of the strains obtained in this study would consequently aid towards the development of bioactive, pharmaceutically and commercially important antimicrobial compounds. Therefore, further studies towards this subject are now essential to identify the active compounds produced in order to discover novel drugs with antimicrobial activity.

## Conclusion

The study revealed that *Zanthoxylum oxyphyllum* Edgew., a medicinal plant widely used by the local tribal communities of Assam, are colonized by endophytic fungi. The dominant endophytes were fungi belonging to genus *Colletotrichum* and *Mycelia sterilia*. The crude extracts obtained from the endophytic fungal isolate belonging to genus *Colletotrichum* and the species *Fusarium oxysporum* showed significant antimicrobial activity against all the selected clinically significant human test pathogens. Principal Component Analysis (PCA) indicated the crude extracts of the isolates belonging to *Colletotrichum gloeosporoides*, *C. boninense* and *C. siamense* showed positive and maximum inhibition against the *S. aureus*. Similarly, isolates *F. oxysporum*, *C. asianum* and *C. nymphae* showed positive and better antimicrobial potential against *C. albicans*. Further detailed investigation on the bioactive metabolites obtained from these endophytic fungal species would therefore lead to the discovery of efficient antimicrobial compounds with wide therapeutic applications.

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## Authors' contributions

RT carried out all the experiments. KT formatted the manuscript, designed, hypothesized the experiment and over all wrote the manuscript.

## Conflict of interests

The authors announce no conflict of interests regarding the publication of this paper.

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