Advances in Traditional Medicine (2020) 20:419–425
https://doi.org/10.1007/s13596-019-00422-9

RESEARCH ARTICLE

Antimicrobial activity of endophytic fungi isolated from the mangrove plant *Sonneratia apetala* (Buch.-Ham) from the Sundarbans mangrove forest

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Received: 29 October 2019 / Accepted: 10 December 2019 / Published online: 9 January 2020

Abstract

Endophytic fungi reside in the intercellular space of plant nourished by the plant. In return, they provide bioactive molecules which can play critical roles on plant defense system. Fifty six endophytes were isolated from the leaves, root, bark and fruits of *Sonneratia apetala*, a pioneer mangrove plant in the Sundarbans, Bangladesh. A total of 56 isolates were obtained and 12 different species within 8 genera were identified using morphological and molecular characteristics. Antimicrobial activity of ethyl acetate (EtOAc) and methanolic (MeOH) extracts of these 12 different species were analyzed by resazurin assay and the minimum inhibitory concentrations (MICs) were determined. The fungal extracts showed antimicrobial activities against more than one tested bacterium or fungus among 5 human pathogenic microbes, i.e. *Escherichia coli* NCTC 12241, *Staphylococcus aureus* NCTC 12981, *Micrococcus lutus* NCTC 7508, *Pseudomonas aeruginosa* NCTC 7508 and *Candida albicans* ATCC 90028. Overall, methanolic extracts showed greater activity than that of ethyl acetate extracts. Of the isolates identified, *Colletotrichum gloeosporioides*, *Aspergillus niger* and *Fusarium equiseti* were the most active isolates and showed activity against microorganisms under investigation. Methanolic extracts of *C. gloeosporioides* and *A. niger* showed the lowest MIC (0.0024 mg/mL) against *P. aeruginosa*. The study indicates that endophytic fungi isolated from *S. apetala* species possess potential antimicrobial properties, which could be further investigated.

Keywords Antimicrobial · Endophytic fungi · Mangrove plants · *Sonneratia apetala* · The sundarbans

Introduction

The appearance of new diseases such as Influenza, SARS, and H1N1 has become a foremost challenge to the human health. Majority of these newly emerged diseases are caused by microorganisms and causative microbes are increasingly becoming drug resistant over time (Bhatia and Narain 2010). To combat such infectious diseases, novel bioactive compounds from plants and microorganisms could provide the best alternative as source of potential and promising drugs (Morens et al. 2004). Although, plant is the major source of bioactive compounds, nevertheless, endophytes could play a vital role in search of new bioactive compounds (Jalgaonwala et al. 2011). Endophytic fungi colonize healthy plant tissues without any disease symptoms and protect plants from herbivores and pathogens as because they produce bioactive secondary metabolites in the host tissues. It is reported that, endophytic fungi could produce similar or the same bioactive metabolites as its host plant. The

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environmental conditions of plant habitat have also some effects on the endophytes present inside the plants (Sette et al. 2006).

Mangroves provide a unique and dominant ecosystem comprised of intertidal marine plants, mostly trees, predominantly bordering margins of tropical coastlines around the world. These halophytic (salt tolerant) plants thrive in intertidal areas that receive daily inundation between mean sea levels and the highest astronomical tides. It has been well established that environment where the plant grows has enormous effect on the diversity of the metabolites produced by the entophytes (Tomlinson 1986; Duke et al. 1998). With this end in view, *Sonneratia apetala* (Buch.-Ham), has been selected for the present study because this plant has hardly been considered for its bioactive endophytic fungi and is one of the most dominant species in the Sundarbans mangrove forest. Thus, the present study was conducted to investigate the abundance of endophytic fungi associated with *S. apetala* found in the Sundarbans, Bangladesh and evaluate antimicrobial activity of the fungal endophytes against human pathogens. Screening of endophytic fungi with inhibitory activities against pathogenic bacteria or fungi might lead to potential novel natural products with higher antimicrobial activity.

Materials and methods

Collection of plant samples

Healthy roots, bark, fruits and leaves of *S. apetala* without any sign of infections were collected from the mangrove forest, the Sundarbans, Khulna, Bangladesh. During sample collection, the plants were identified based on their morphological characteristics outlined by Zabala (1990).

Isolation of endophytic fungi

Isolation of endophytic fungi was carried out according to the procedure described by Nurunnabi et al. (2018). Briefly, plant materials were washed thoroughly in sterile water to remove extraneous substances, followed by surface sterilization (sequentially immersing in 70% ethanol for 30 s and 5% sodium hypochlorite solution for 1 min) and finally rinsed with sterile distilled water. The samples were surface-dried with sterile filter paper (Hormazabal and Piontelli 2009). Roots, bark, fruits and leaves were cut into 0.5 cm × 0.5 cm pieces and placed in petri dishes containing aqueous agar (1.5% agar–agar in distilled water) supplemented with antibiotic streptomycin (0.03 mg/mL) and incubated at 28 ± 2 °C until initiation of fungal growth. The tips of the fungal hyphae were collected from the aqueous agar and placed on potato dextrose agar (PDA) medium. After several days of incubation, the purity of each fungal culture was determined by assessing the colony morphology. The isolates were cultured on PDA media to obtain pure cultures and then transferred into PDA slants for preservation at 4 °C.

Microscopic and molecular identification of the fungal isolates

Both morphological and molecular techniques were employed to identify the isolated endophytic fungi. Morphological identification was performed according to the procedure described by Qadri et al. (2013). Fungal genomic DNA was isolated according to the protocol supplied along with the DNA isolation kit (Cat-26200; NORGEN BIOTEK Corp., 3430 Schmon Parkway, Thorold, ON, Canada). Forward primer ITS4 (5′TCCTCCGCTTATTGATATGC3′) and reverse primer ITS5 (5′GGAAGTAAAAGTCGTAACAAGG3′) were used to amplify the internal transcribed spacer (ITS) region of the fungi using Polymerase Chain Reaction (Sette et al. 2006). The PCR reaction was performed in 50 µl reaction volume comprising 10 µl of 5 × reaction buffer, 3 µl of 1.5 M $MgCl_2$, 5 µl of 10 mM dNTP, 3 µl of 100 pmol primers, 26 µl of distilled water and 3 µl (2 ng/ µl) of template DNA. The PCR amplification was performed using 2720 PCR Thermo Cycler and the temperature profile was maintained as initial denaturation at 94 °C for 5 min followed by 40 cycles (denaturation 94 °C for 30 s, annealing 51 °C for 30 s and extension 72 °C for 2 min). The final extension was carried out at 72 °C for 5 min and held at 4 °C. The PCR amplification of the ITS region was verified by electrophoresis (10 µL) and the rest of the PCR products (40 µl) were purified according to requirement of Cambridge Genomic Services (The University of Cambridge, UK) for DNA sequencing. Multiple BLASTN searches against the sequence were made at the National Center for Biotechnology Information (NCBI). Consensus sequences were submitted in the GenBank. Primarily, Clustal Omega was employed for Multiple Sequence Alignment and further followed by trimming using trimAI tool for later alignment. Phylogenetic analysis performed with the neighbor joining method using MEGA7.0 software.

Extraction of secondary metabolites

Extraction of the secondary metabolites was carried out according to the Nurunnabi et al. (2018). The fungal isolate was grown in (5 × 250 ml) conical flasks containing potato dextrose broth (PDB) for 28 days. Culture broth was separated from the mycelium by filtration (Whatman® qualitative filter paper, Grade 1; Sigma-Aldrich, USA) and the filtrates were extracted three times with an equal volume of ethyl acetate (EtOAc) in a separating funnel. The mycelium was dissolved in methanol under dark condition for two days and

the mycelium was separated by filtration. Similarly, methanolic extracts were also prepared and both types of extracts were evaporated under reduced pressure at 40–45 °C using a rotary evaporator to obtain crude extracts.

Antimicrobial screening

The EtOAc and Methanolic extract of all 12 isolated fungi were tested for their potential antimicrobial activity against two Gram-positive, i.e., *Staphylococcus aureus* NCTC 12981 and *Micrococcus luteus* NCTC 7508, and two Gram-negative, i.e., *Escherichia coli* NCTC 12241 and *Pseudomonas aeruginosa* NCTC 12903 bacterial strains as well as against a fungal strain, *Candida albicans* ATCC 90028 using the resazurin 96-well microtitre plate based in vitro antimicrobial assay (Sarker et al. 2007) For bacterial strains, Ciprofloxacin was used as a positive control and nystatin for *C. albicans*. Resazurin solution (4 mg of resazurin was dissolved in 20 mL of sterile distilled water) was used in this assay as an indicator of cell growth. Briefly, sterile 96 well plates were prepared and labeled under aseptic conditions. A volume of 100 µl of test material in 10% (v/v) DMSO (10 mg/ml for crude extracts) was pipetted into the first row of the plate and 50 µl of normal saline was added to all other wells. Serial dilutions were performed in such that each well had 50 µl of the test material in serially descending concentrations. 30 µl nutrient broth and 10 µl of resazurin indicator solution were added to each well. Finally, 10 µl of bacterial suspension (5×10^5 cfu/ml) was added to each well. To prevent the bacterial culture dehydration, each plate was wrapped loosely with cling film. Each plate had a column with a broad-spectrum of antibiotic as positive control (usually ciprofloxacin in serial dilution) a column with all solutions with the exception of the bacterial solution adding 10 µl of nutrient broth instead. The plates were prepared in triplicates, and placed in an incubator set at 37 °C for 18–24 h. The color change was then assessed visually. Any color changes from purples to pink or colorless were recorded as positive. The lowest concentration at which color change occurred was taken as the minimum inhibitory concentration (MIC) value. The average of three values was calculated and that was the MIC for the test materials.

Calculation and statistical analysis

Isolation rate (IR) was calculated using the formula of Gong and Guo (2009). Isolation rate (IR) was used to demonstrate the fungal richness in a given sample of plant tissue which was counted as the number of isolates obtained from plant segments divided by the total number of segments incubated. The statistical analysis was analyzed using Graph Pad Prism, version 6.0.

$$\mathrm{IR} = \frac{\text{the total number of isolates yielded in a given trial}}{\text{The total number of samples in that trial}} \quad (1)$$

Colonization rate (CR) expressed in percentage, was calculated as the total number of Colonization rate (CR), expressed in percentage, which was calculated as the total number of plant tissue segments infected by fungi divided by the total number of segments incubated, was used to indicate comparison of degrees of different tissues infected by endophytic fungi. Isolation rate (IR) was used to demonstrate the fungal richness in a given sample of plant tissue which was counted as the number of isolates obtained from plant segments divided by the total number of segments incubated (Gong and Guo 2009).

Results

Isolation and identification of endophytic fungi

A total of 58 endophytic fungi were isolated from 120 tissue segments (30 segments from Root, Bark, Leaves and Fruits each) from *S. apetala*. The isolation rates for Root, Bark, Fruits and Leaves were 46, 43, 33 and 70%, respectively. The isolation rates of endophytic fungi from leaves were higher than that of bark, root and fruits. On the basis of morphological analysis (Fig. 1), 12 different types of endophytes were selected for molecular identification. Genomic DNA of all 12 endophytic fungi was extracted and multiplied by ITS4 and ITS5 universal primers. After gel electrophoresis, the PCR amplified products of abound 500 bases were obtained and presented in Fig. 2.

DNA sequences obtained from amplification of ITS region were submitted to Genbank and accession numbers for each fungus were obtained. A range of 520 to 602 nucleotide pair sequences were obtained. *Neopestalotiopsis chrysea* has the shortest length of ITS region of 520 base pair in contrast to *Aspergillus niger* which was 602 base pair. Table 1 represents the sequence data of isolated endophytes from *S. apetala*, where 99 to 100% BLAST match sequences were obtained.

Pair wise genetic distance and phylogenetic tree construction

Table 2 represents the pair-wise genetic distances estimated from sequenced data. The pair-wise genetic distances ranged from 0.376 to 0.927. The maximum pair-wise genetic distance (0.927) was found between SaL11 (*A. niger*) and SaR7 (*F. equiseti*). The result can be justifying by reason that they both are from different genus. Although three *Alternaria* spp. and two *Cladosporium* species were present but the

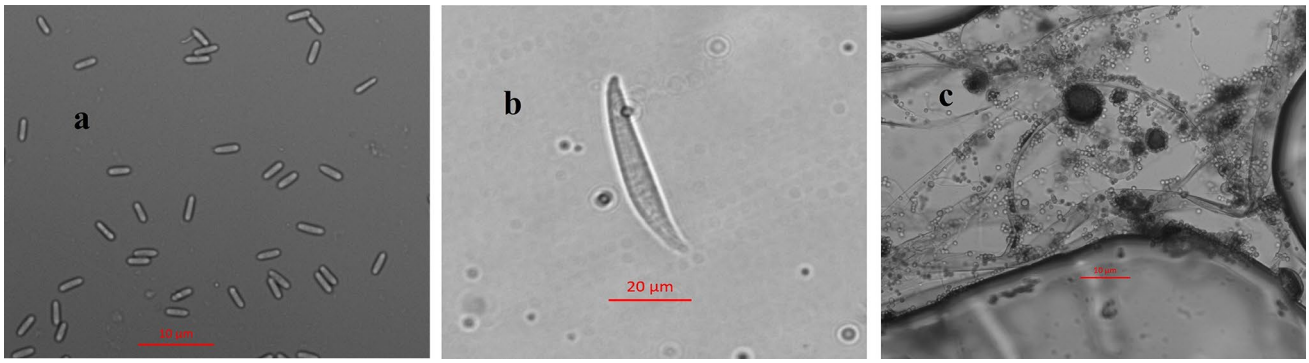

Fig. 1 Light micrographs of endophytic fungi isolated from *S. apetala.* **a** *Colletotrichum glosporides*. **b** *Fusarium equiseti*. **c** *Aspergillus niger*

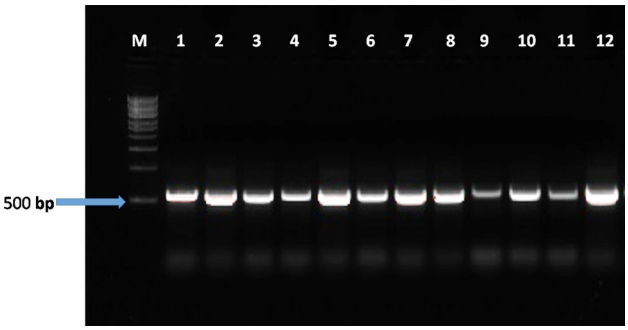

Fig. 2 PCR amplified products of ITS region of Endophytic fungi isolated from *S. apetala* (M = size marker; 1 = SaR7; 2 = SaL4; 3 = SaL2; 4 = SaL5; 5 = SaR9; 6 = SaL13; 7 = SaF1; 8 = SaF5; 9 = SaL9; 10 = SaKB6; 11 = SaR1; 12 = SaL11)

maximum similarity was found in between SaL5 (*Alternaria arborescens*) and SaF5 (*Epicoccum nigrum*). A UPGMA tree was constructed (Fig. 3) from these distance matrix using the software PUAP 4.0. The UPGMA tree of 12 isolated endophytic fungi shows two main clusters. Cluster one consisted with eight fungi viz. SaL11 (*A. niger*), SaL2 (*Alternaria brassicicola*), SaR7 (*F. equiseti*), SaR9 (*Cladosporium cladosporioides*), SaR1 (*Xenoacremonium recifei*), SaL13 (*Cladosporium perangustum*), SaL9 (*Colletotrichum glosporides)* and SaF1 (*Cladosporium tenuissimum)*. The second cluster was consisted with four fungi and they are SaL5 (*A. arborescens*), SaF5 (*E. nigrum*), SaL4 (*A. tenuissima*) *and* SaB6 (*Neopestalotiopsis chrysea*).

Antimicrobial activities of ethyl acetate and methanolic extracts

Methanolic Extracts from *C. cladosporioides*, *C. perangustum C. tenuissimum, F. equiseti, A. brassicicola, A. arborescens, C. glosporides, N. chrysea* and *A. niger* showed effect on all the tested microorganisms and for the Ethyl Acetate extract *F.equiseti, C. glosporides* and *A. niger* showed activity against all tested microorganisms (Table 3). On average, the methanolic extracts seem to be the more effective on the tested microorganisms. Methanolic and Ethyl Acetate extract from *F. equiseti*, *C. glosporides* and *A. niger* showed comparatively greater antimicrobial activity against all tested microorganisms. Methanolic extracts from *C. glosporides* and *A. niger* confirm the highest MIC (0.00024 mg/ml) for *S. aureus* (NCTC 12981). All the fungal extract showed activity against *P. aeruginosa* (NCTC 12903), *S. aureus* (NCTC 12981) and *M. luteus* (NCTC 7508) extracts showed lesser activity against *E. coli* (NCTC 12241) and *C. albicans* (ATCC 90028), respectively.

Discussion

The present study has revealed that *S. apetala* harbors a lot of endophytic fungi that produces potent antimicrobial substances. The findings of this study also suggest that endophytes from harsh and competitive environments, such as mangrove ecosystem, might be an attractive source for new anti-infective compounds. Xing et al. has recently reported *Mycosphararella* spp., *Fusarium* spp. *Glomerella* spp., *Phomopsis* spp., and *Pestalotiopsis microspore* from *S. apetala* (Xing et al. 2011). The present work is the first report of endophytic fungi *A. tenuissima, A. brassicicola, A. arborescens, C. cladosporioides, C. perangustum, C. tenuissimum, E. nigrum, N. chrysea, X. recifei and A. niger isolated from S. apetala.* In the present study, *Fusarium* sp. inhibited both bacteria and fungi with lowest MIC, which was in agreement with the information reported in a previous study that demonstrated *Fusarium* sp. isolated from *Saussurea involucrata* with broad antimicrobial spectrum (Lv et al. 2010). In another study, endophytic fungi isolates from twelve mangrove species were found to be diverse. Similar to our study, the study reported the extraction of fungal broth by ethyl acetate while the mycelia were extracted using methanol, hexane and ethyl acetate in sequence, respectively (Buatong

Table 1 Identification of endophytic fungi in *Sonneratia apetala*

Internal code	Name of the fungi	Genbank accession number	BLAST match sequence		
			Reference accession number	Query length	Similarity (%)
SaR7	*Fusarium equiseti*	MH128127.1	*Fusarium equiseti* HM008677.1	529	99
SaL4	*Alternaria tenuissima*	MH127848.1	*Alternaria tenuissima* KT223327.1	552	99
SaL2	*Alternaria brassicicola*	MH203590.1	*Alternaria brassicicola* KY310727.1	565	99
SaL5	*Alternaria arborescens*	MH145449.1	*Alternaria arborescens* KT223325.1	549	100
SaR9	*Cladosporium cladosporioides*	MH124140.1	*Cladosporium cladosporioides* KY921931.1	532	99
SaL13	*Cladosporium perangustum*	MH145453.1	*Cladosporium perangustum* HM148148.1	532	99
SaF1	*Cladosporium tenuissimum*	MH145451.1	*Cladosporium tenuissimum* KY921930.1	535	99
SaF5	*Epicoccum nigrum*	MH145450.1	*Epicoccum nigrum* KM519661.1	527	99
SaL9	*Colletotrichum glosporides*	MH150838.1	*Colletotrichum glosporides* MF838770.1	550	99
SaB6	*Neopestalotiopsis chrysea*	MH145454.1	*Neopestalotiopsis chrysea* KU534877.1	520	99
SaR1	*Xenoacremonium recifei*	MH150837.1	*Xenoacremonium recifei* KM231834.1	544	99
SaL11	*Aspergillus niger*	MH447401.1	*Aspergillus niger* KF305758.1	602	100

Table 2 Pairwise genetic distance of the endophytic fungi isolated from *S. apetala*

	SaL11	SaR7	SaR9	SaR1	SaL13	SaL9	SaL5	SaL4	SaL2	SaF5	SaF1	SaB6
SaL11	–											
SaR7	0.927	–										
SaR9	0.730	0.705	–									
SaR1	0.727	0.695	0.713	–								
SaL13	0.752	0.738	0.690	0.635	–							
SaL9	0.732	0.744	0.729	0.697	0.701	–						
SaL5	0.771	0.740	0.783	0.732	0.725	0.742	–					
SaL4	0.769	0.756	0.730	0.756	0.734	0.787	0.734	–				
SaL2	0.686	0.705	0.752	0.764	0.789	0.754	0.709	0.730	–			
SaF5	0.768	0.748	0.771	0.738	0.715	0.752	0.376	0.697	0.732	–		
SaF1	0.744	0.713	0.746	0.699	0.752	0.491	0.748	0.785	0.748	0.742	–	
SaB6	0.775	0.744	0.755	0.740	0.779	0.752	0.752	0.707	0.720	0.759	0.742	–

Sa, *Sonneratia apetala*; L, Leaves; R, Root; F, Fruit; B, Bark

et al. 2011). Ethyl acetate is widely used in extraction of endophytic fungal cultures (Bhardwaj et al. 2015; Minarni et al. 2017) followed by methanol. As a solvent, ethyl acetate solvent possesses medium polarity so that it has the ability to dissolve both polar and non-polar active compounds and methanol solvent being a polar solvent can dissolve almost all organic compounds, even polar, semi polar and non-polar (Rahmawati et al. 2018). However, apart from separation of compounds, the solvents had no effect on antimicrobial potential of the fungal extracts as the extracts were evaporated under reduced pressure using rotary evaporator.

In the present study, we have identified the endophytic fungus through morphological analysis, however, this type of identification is presumptive and requires fair bit of experience. Therefore, in addition to this, we chose nucleotide sequence analysis of ITS region because it lies between two

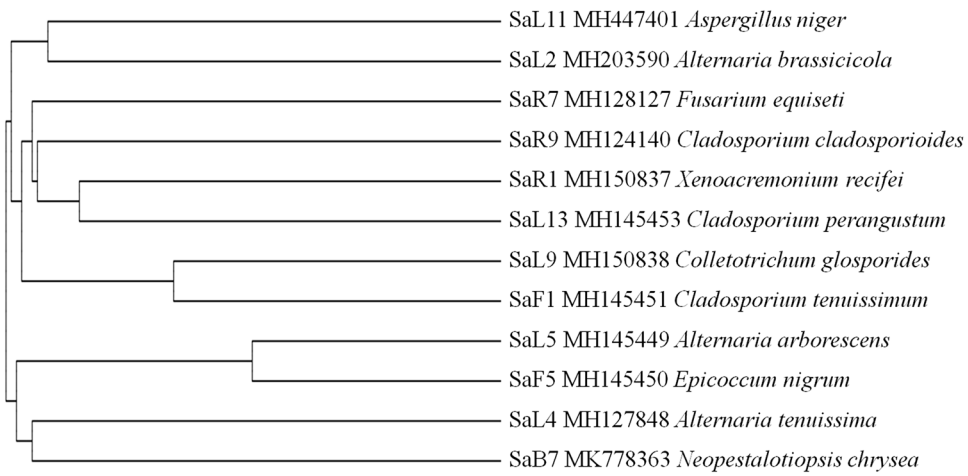

Fig. 3 Phylogenetic tree, constructed based on rDNA sequence (ITS1, 5.8S and ITS 4) by using UPGMA. The bootstrap consensus tree inferred from 1000 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option)

Table 3 Minimum inhibitory concentration (MIC) mg/ml of ethayl acetate and methanolic extract of 12 different endophytic fungi

Name of the fungi	Extract	*E. coli* (NCTC 12241)	*M. luteus* (NCTC 7508)	*P. aeruginosa* (NCTC 12903)	*S. aureus* (NCTC 12981)	*C. albicans* (ATCC 90028)
Cladosporium cladosporioides	EtOAc	NA	2.5	2.5	5	NA
	MeOH	5	2.5	2.5	2.5	10
Cladosporium perangustum	EtOAc	NA	2.5	2.5	5	NA
	MeOH	5	5	2.5	5	10
Cladosporium tenuissimum	EtOAc	10	2.5	2.5	5	NA
	MeOH	5	2.5	2.5	2.5	10
Fusarium equiseti	EtOAc	5	0.0097	1.25	0.0097	2.5
	MeOH	2.5	0.0097	0.062	0.0097	2.5
Alternaria tenuissima	EtOAc	0.125	0.125	0.0097	0.0097	NA
	MeOH	0.062	0.062	0.0097	0.062	NA
Alternaria brassicicola	EtOAc	NA	0.125	0.062	0.062	2.5
	MeOH	0.062	0.062	0.0097	0.062	0.062
Alternaria arborescens	EtOAc	NA	0.125	0.0097	0.0097	2.5
	MeOH	0.062	0.062	0.0097	0.062	0.062
Epicoccum nigrum	EtOAc	NA	2.5	10	5	NA
	MeOH	10	0.0097	2.5	5	NA
Colletotrichum glosporides	EtOAc	5	0.25	0.125	0.25	2.5
	MeOH	0.25	0.062	0.00024	0.125	0.062
Neopestalotiopsis chrysea	EtOAc	0.25	0.062	0.0097	0.125	NA
	MeOH	5	10	2.5	5	0.062
Xenoacremonium recifei	EtOAc	NA	2.5	1.25	5	NA
	MeOH	NA	1.25	2.5	5	NA
Aspergillus niger	EtOAc	0.25	0.062	0.0097	0.062	0.125
	MeOH	0.062	0.062	0.00024	0.062	0.125
Ciprofloxacin		4.9×10^{-4}	9.8×10^{-4}	1.2×10^{-4}	9.8×10^{-4}	NT
Nystatin		NT	NT	NT	NT	9.8×10^{-4}

NA, not activity; NT, not tested

highly conserved genes coding for 18S and 28S rRNA. The ITS regions circumscribe two non-coding regions ITS1 and ITS2, which are separated by the highly conserved 5.8S rRNA gene (Crouch et al. 2009). The ITS1 and ITS2 regions are more variable than the other adjacent rRNA gene sequences (Ciardo et al. 2006). Therefore, offer a better

identification of closely related species. By sequence determination and comparison with the sequences of Genbank data base, all the strains identified to the species level (99 to 100% homology to the best-matching reference sequence).

In this study, we successfully isolated and identified 12 different species of endophytic fungi belonging to 8 different genera. All the isolates are moderately active against tested microorganisms. However, further studies could be initiated with *F. equiseti*, *C. glosporides* and *A. niger* for potential bioactive compounds as our results showed promising activities with these endophytic fungi. The findings of this study also suggest that endophytes from harsh and competitive environments, such as mangrove ecosystem, might be an attractive source for bio-prospecting of new antimicrobial compounds.

Acknowledgements The research was funded by Robert S. McNamara Fellowship Programme from the World Bank.

Compliance with ethical standards

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest Tauhidur R. Nurunnabi has no conflict of interest. Farah Sabrin has no conflict of interest. Dilara I. Sharif has no conflict of interest. Lutfun Nahar has no conflict of interest. Md. H. Sohrab has no conflict of interest. Satyajit D. Sarker has no conflict of interest. S. M. Mahbubur Rahman has no conflict of interest. Md. Morsaline Billah has no conflict of interest.

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