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Short Communication

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Bioactive potential of endophytic fungi isolated from *Phyllanthus niruri* L.

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Abstract

Present work describes the isolation and morphological identification of endophytic fungi from *Phyllanthus niruri* L. including their chemical and bioactivity screening. Two isolated and purified endophytic fungi with the internal strain numbers PNRE and PNLE were taxonomically identified as *Colletotrichum* sp. and *Fusarium* sp., respectively. Preliminary chemical screening of the fungal extracts through thin layer chromatography (TLC) revealed the presence of various components. PNLE showed significant antifungal activity against the fungi *Aspergillus niger* (24 mm) and *Aspergillus flavus* (20 mm). Extracts of PNRE and PNLE gave significant cytotoxic activity with LC_{50} values of 1.08 µg mL^{-1} and 1.05 µg mL^{-1}, respectively in the brine shrimp lethality bioassay. PNRE extract showed mild scavenging of DPPH with IC_{50} value of 96.06 µg mL^{-1} during antioxidant activity screening. Crystal formation was observed in the crude PNRE fungal extract which was purified using methanol/dichloromethane (0-20%) to obtain pure white crystal and the structure was elucidated by NMR (^{1}H NMR and ^{13}C NMR) spectroscopic analysis to be confirmed as ergosterol. Since isolated fungi showed promising antifungal and cytotoxic activities, the crude extract of endophytic fungi of *P. niruri* could be considered as a promising source for isolation of potential bioactive compounds.

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Introduction

Plants have a long history of traditional medicinal applications which are well established through the pharmacological investigation of the plant and its derived compounds (Gorniak *et al.*, 2018). Endophytic fungi have been isolated from various plants studied to date including trees, grass, algae, and herbaceous plants. Khan *et al*. (2018) isolated endophytic *Fusarium solani* and found it as a rich source of cytotoxic and antimicrobial napthaquinone and aza-anthraquinone derivatives. Endophytic fungi inhabiting the tissues of the host plant for a certain phase in their life cycle cause no apparent harm to the host plant (Yu *et al.*, 2010). They develop a mutualistic relationship with the host by protecting it against pathogen and thus, may term as an opportunistic symbiont (Sultan *et al.*, 2011). Most endophytes are capable of synthesizing rare and novel compounds with important pharmacological activities including anti-tumor and anti-microbial activities, which may provide plants with a resistance against pathogens. Some of these compounds have proven useful for novel drug discovery (Hoque *et al.*, 2018; Khan *et al.*, 2018; Guo *et al.*,

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2008; Yan *et al.*, 2011; Chen *et al.*, 2016). Some endophytic fungi developed the ability to produce the same or similar bioactive substances as those originated from their host plants (Shah *et al.*, 2017). Although endophytes providing a rich and reliable source of genetic diversity and biological innovation can be applied in pharmacology and agriculture, very few of them have been cultured and screened for drugs (Strobel and Daisy, 2003; Wu *et al.*, 2015). Moreover very few medicinal plants from Bangladesh have been investigated for their endophytic fungal diversity (Tanvir *et al.*, 2017). There have been reports on isolation of endophytic fungal strains of *Colletotrichum* sp., *Phoma* sp., *Phyllosticta* sp., *Penicillium* sp., *Pestalotiopsis* sp., *Fusarium* sp., *Altermeria* sp., and *Edenia* sp. from different plant parts of *P. niruri* collected from India (Kandasamy *et al.*, 2015; Kandavel and Sekar, 2015).

More than 50 compounds were reported from *P. niruri* (Bagalkotkar *et al.*, 2006). *P. niruri* is used in efficient treatment of asthma, stimulating liver, improving digestion, increasing appetite and also produce laxative effects (Narendra *et al.*, 2012). It is bitter in taste but sweet in the post digestive effect and used as astringent (Narendra *et al.*, 2012). Indeed, the aqueous and methanol extracts of *P. niruri* expressed an inhibitory effect against the human melanoma (MeWo), prostate cancer (PC-3), lung cancer (A549) and breast cancer cell line (MCF-7) (Tang *et al.*, 2010; Lee *et al.*, 2011).

In this work we have isolated and characterized the endophytic fungi of *P. niruri* growing in Bangladesh, with the aim to establish a repository of fungal diversity and screened their bioactivity relative to the previously reported results of crude plant extract.

Materials and methods

Collection of samples and isolation of endophytic fungi

The plant samples were collected from the botanical garden of Bangladesh Council of Scientific and Industrial Research, Dhaka, Bangladesh. About 300 g of fresh and healthy parts of the plant (leaf and root) were cut with a sterile scalpel and stored at 4°C in a sterile polythene bag prior to use. Endophytic fungi were isolated from this fresh and healthy plant parts through a moderately modified method by Kusari *et al.* (2009). The leaves and root parts were thoroughly washed in running tap water followed by distilled water and then they were taken into sterile condition of laminar flow cabinet (Esco AHC4DI; UK). Leaf fragments with 3-5 leaves each of approximately 4-5 × 3-5 mm (length × width) size and root segments of approximately 15-20 mm were cut with sterile razor blade. Surface sterilization was performed by sequential immersion of the sample in 70% ethanol for 1 min, 1.3 mol L^{-1} sodium hypochlorite (approximately 5% available chlorine) for 3 min and 70% ethanol for 30 sec. The sample was finally washed in sterile distilled water three times for 1 min each. After excess moisture was removed, the plant parts were inoculated in Petri dishes containing water agar (WA) media amended with streptomycin (100 mg L^{-1}). Petri dishes were sealed using Parafilm (Parafilm M, Malaysia) and incubated at 28 ± 2°C in an incubator (Froilabo, BCR 120, UK) for 21 days. To ensure isolation of endophytes, unsterilized respective plant parts were prepared simultaneously by washing only with sterile distilled water to isolate the surface-contaminating fungi and incubated under the same conditions in parallel. The cultures were monitored every 2-3 days to check the growth of endophytic fungal hyphae. As the hyphal tips of the fungi appeared from the plant part in the isolation media they were transferred onto potato dextrose agar (PDA) media and serial dilution technique was used on PDA to obtain pure fungal colonies.

Endophytic fungal identification

The fungi were identified using relevant keys and taxonomic notes from various standard manuals (Barnett and Hunter, 1998). Slides of fungal preparations were stained with lactophenol cotton blue reagent and then examined with a bright-field and phase contrast microscope (Sadananda *et al.*, 2014). Colony description was done through periodic examination of some morphological characteristics such as growth pattern, hyphae, the color of the colony and medium, surface texture, margin character, aerial mycelium, sporulation and production of acervuli, coloration of the medium, and the size and coloration of the conidia of the subcultures in PDA media using standard identification manuals (Devi and Prabaharan, 2014).

Endophytic fungal cultivation and extraction

Small scale cultivation was performed on Petri dishes for the isolated fungal strains with approximately 1 L of PDA media for each strain. Endophytic isolates were incubated for 21 days at 28ºC and the culture media were extracted two times with ethyl acetate to obtain the crude extracts (Chowdhury *et al.*, 2016). The extracts of the fungi were concentrated into solid residue by evaporation with rotary vacuum evaporator under reduced pressure (Alzoreky and Nakahara, 2003) to obtain ethyl acetate crude extract.

Chemical screening

The endophytic fungal crude extracts were subjected to TLC for initial screening of its chemical constituents as per

established literature (Wagner and Bladt, 1995) using commercially available precoated silica gel (kieselgel 60 PF254) plates with solvent system of Toluene:10% Ethyl acetate.

Antimicrobial activity assay

The fungal crude extracts were screened for their antimicrobial activity by slight modification of the disc diffusion method using four pathogenic bacterial strains *Staphylococcus aureus* ATCC 25923*, Escherichia coli* ATCC 25922*, Bacillus megaterium* ATCC 13578 and *Pseudomonas aeruginosa* ATCC 27833, two fungal strains *Aspergillus niger* and *Aspergillus flavus* (Bauer *et al.*, 1966). Kanamycin and Ketoconazole sensitivity disc (30 µg/disc) were used as positive control for bacteria and fungi, respectively. Solvents were used as negative control. The sensitivities of the microorganism species to the fungal extract (100 µg/disc) were determined by measuring the diameter of inhibitory zones in millimeter after 24 h of incubation at 37°C for bacteria and 96 h of incubation at 28°C for fungi.

Antioxidant activity assay

Antioxidant activities of the endophytic fungal samples were assayed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method (Brand-Williams *et al.*, 1995). Here, 2 ml of various concentrations (800 – 6.25 µg ml^{-1}) of extracts were mixed with 2.0 ml of 0.05 mM DPPH solution. The mixture was shaken and left for 30 min in dark and the absorbance was measured spectroscopically at 517 nm. The scavenging activity was calculated as follows:

$$\frac{(Absorbance\,of\,control - Absorbance\,of\,sample)}{Absorbance\,of\,control} \times 100$$

For each sample, the result was presented as an IC_{50}. Ascorbic acid (AA) and 2-tert-butyl-4-hydroxyanisole (BHA) were used as positive control.

Cytotoxicity assay

For the brine shrimp lethality bioassay, the eggs of brine shrimp nauplii (*Artemia salina*) were commercially obtained from a pet shop, hatched for 24 hours and tested for LC_{50} values against varying concentration (200 – 0.39 µg ml^{-1}) of fungal extracts in dimethylsulphoxide (DMSO) obtained by the serial dilution technique (Meyer *et al.*, 1982; Ahmed *et al.*, 2010; Rahman *et al.*, 2012). Vincristive sulphate was used as positive control and DMSO was used both as a solvent and negative control. All the samples were tested in triplicate.

Isolation of compound and characterization using NMR spectral analysis

Crystal formation was observed in the ethyl acetate crude extract of PNRE fungal strain which was purified by solvent treatment using methanol followed by methanol/(0-20%) dichlomethane mixture which lead to pure white crystal (compound **1**). ^{13}C and ^{1}H NMR spectroscopic analyses were performed for the isolate (compound **1**) with a Bruker Advance 400 MHz (Germany) NMR spectrometer using $CDCl_3$ as solvent at 25 °C to elucidate the structure of compound **1** and chemical shifts were reported in ppm.

Results and discussion

Endophytic fungal identification

Two endophytic fungi namely PNRE and PNLE were isolated and purified from the medicinal plant *P. niruri.* Fungi were identified taxonomically to the genus level on the basis of macroscopic and microscopic morphological characters in culture medium as shown in Fig. 1. From microscopic view of PNRE, mycelliums were observed as branched, septate and hyaline. Acervuli were subepidermal. Conidiophore was simple, elongated, disc shaped or cushon shaped. Conidia were hyaline, one celled, ovoid to oblong. These characteristics indicate that the PNRE strain belonged to the *Colletotrichum* genera (Barnett and Hunter, 1998). Mycelliums of PNLE strain were branched, septate and hyaline. Conidiophore was slender and simple. Macrconidia were hyaline, two to three celled, slightly curved. Microconidia were one celled and oblong. Chlamydospore was present. These characteristics suggest that PNLE strain was from *Fusarium* genera (Barnett and Hunter, 1998).

Chemical screening

Endophytes are known to synthesize different groups of compounds that protect plant against pathogen and some of them have been proven to have different pharmacological activities (Hoque *et al.*, 2018; Guo *et al.*, 2008). All extracts were qualitatively tested for the presence of chemical constituents by TLC technique. TLC results indicate that PNRE strain may contain coumarin and isocoumarin; and flavonoids, anthraquinine and napthaquine components could be present in PNLE extract (Wagner and Bladt, 1995). Large scale cultivation of the fungi aiming at bulk isolation of the compounds on the basis of their chemical screening will help explore their competence as leads for drug discovery.

Antimicrobial activity assay

In the preliminary antimicrobial screening, no antibacterial activities were observed for the fungi strains PNRE and PNLE. Only PNLE showed good antifungal activity against *Aspergillus niger* (24 mm) and *Aspergillus flavus*, (20 mm) as compared with the positive control ketoconazole which is

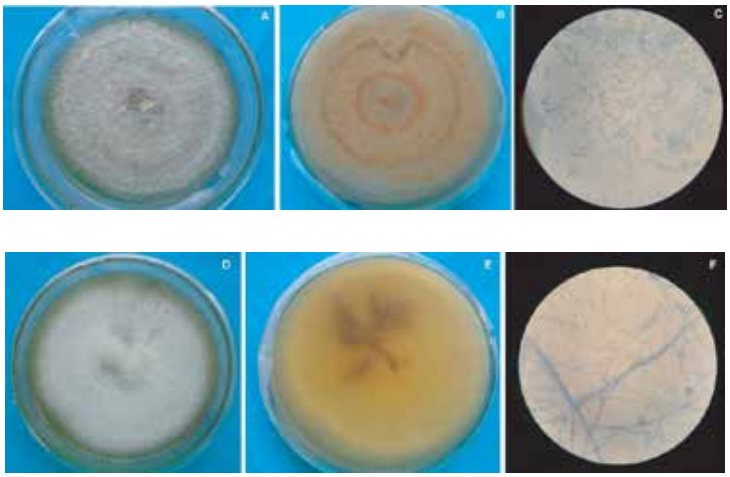

Fig. 1. Colony morphologies of the endophytic fungi from *Phyllanthus niruri* L., Where, A-Upper View, B-Lower View, C-Microscopic View of PNRE *(Colletotrichum sp.);* D-Upper View, E-Lower View, F-Microscopic View of PNLE *(Fusarium sp.)* at 40x

shown in Fig. 2. This result indicates that the endophytic fungus PNLE may produce secondary metabolites with antifungal potential.

Antioxidant activity assay

With different concentration of samples (0.78-200 µg ml^{-1}) the percentage of inhibition of PNRE was 5.44-79.08%; PNLE was 9.14-72.05% whereas the ability to scavenge DPPH free radicals was up to 90% for each standards, as shown in Fig. 3. The IC_{50} value of PNRE and PNLE were 96.06 and 119.72 µg ml^{-1}, respectively where the IC_{50} value of positive control, BHA and AA were 5.94 and 5.51 µg ml^{-1}, respectively. The higher IC_{50} values of the fungal extracts as compared to the standards suggested their weak antioxidant activity (Brighente *et al.*, 2007).

Cytotoxicity assay

Endophytes, isolated from the medicinal plants are reported to be the innovative sources of new drug discovery and development with identical activities as the host plant (Tejesvi *et al.,* 2007). There have been reports on different pharmacological and biological activities of *P. niruri* which prompted the bioactivity screening of its associated fungi (Paithankar *et al.*, 2011). The brine shrimp lethality bioassay, which is an effective and rapid screening method, was applied to determine the general pharmacological activity and toxicity of the endophytic fungal strains isolated from *P. niruri*. With different concentration of samples (0.39 - 200 µg ml^{-1}) the percent of mortality of both PNRE and PNLE were 60-90% after 24 hours of experiment. The LC_{50} values of both PNRE and PNLE were approximately 1.0 µg ml^{-1} (Fig. 4). This result suggests that the fungal extracts may possess

Fig. 2. Activity of PNLE against *Aspergillas niger* and *Aspergillas flavus*. Here, A_2 indicates PNLE extract; Kito indicate Ketoconazole

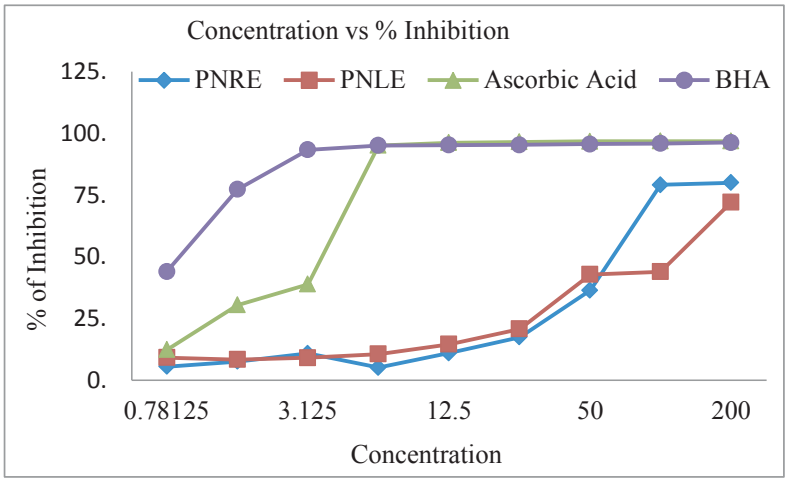

Fig. 3. Free radical scavenging activity of different concentrations of endophytic fungus, PNRE (*Colletotrichum* sp.), PNLE (*Fusarium sp.*) and two standards

different pharmacological activities that can be confirmed with more specific screening.

Isolation and characterization of compound 1 as ergosterol

Compound **1** (Fig. 5) was obtained as white needles with melting point of 149°C. It appeared as a dark quenching spot on the TLC plate (R_f= 0.35, Toluene/20% EtOAc), under UV light at 254 nm. Spraying the developed plate with vanillin-sulphuric acid, followed by heating, at 110°C for several minutes, gave a purple color.

The ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of compound **1** displayed 26 carbon resonances. The ^{13}C NMR spectral data also suggested that compound 1 was a sterol with 6 methyl signals at δ = 12.1, 16.3, 17.6, 19.6, 19.9, and 21.1, an oxygenated methylene signal at δ =70.5 and olefinic carbon signals at δ = 116.3, 119.6, 132.0, 135.5, and 141.3. The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) showed two one-proton multiplets at δ = 3.62 and δ = 5.56, typical for the signals of H-3 and H-6 of a steroidal nucleus. The multiplets at δ = 5.18 and δ = 5.37 could be attributed to three olefinic protons at C-7 and in the side chain, as would be expected for ergosterol. Based on above data and by comparing its

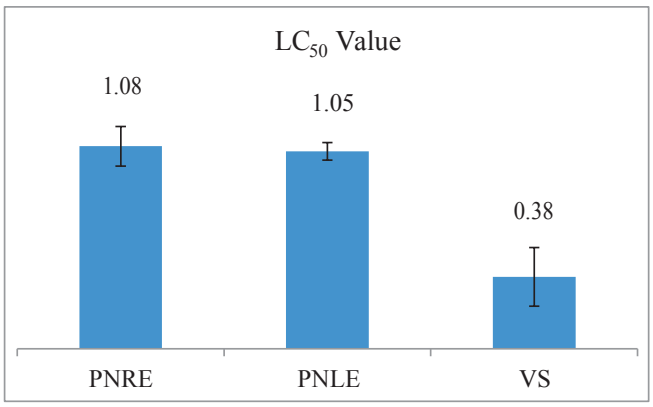

Fig. 4. Brine Shrimp Lethal Bioassay activity of different concentrations of endophytic fungi extract PNRE, PNLE and standard Vincristine sulphate with standard deviation error bar for n=3

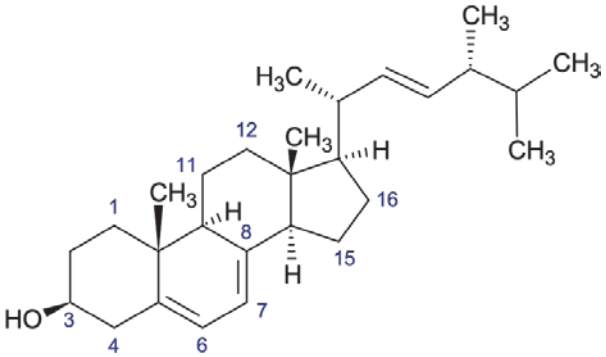

Fig. 5. Ergosterol (1)

spectroscopic data with those reported for the compound in literature (Khan *et al.*, 2018; Martinez *et al.*, 2015; Goulston *et al.*, 1975), compound **1** was identified as ergosterol.

It has been proven that ergosterol possesses a wide spectrum of biological activity. This compound shown to have

Table 1. ^{1}H (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) spectral data of 1, Ergosterol

	$\delta_{H,}$ (1)	δ_H (Khan *et al.,* 2018; Martinez *et al.,* 2015)	$\delta_{C,}$ (1)	δ_C (Khan *et al.,* 2018; Martinez *et al.,* 2015)
1			38.4	38.4
2			32.0	32.0
3	3.62 (1H, m)	3.60-3.66 (1H, m)	70.5	70.4
4			40.8	40.8
5				139.8
6	5.56 (1H, m)	5.55–5.57 (1H, m)	119.6	119.6
7	5.37 (1H, m)	5.37-5.38 (1H, m)	116.3	116.3
8			141.3	141.3
9			46.2	46.2
10			38.0	37.0
11			29.7	21.1
12			39.1	39.1
13				42.8
14			54.5	54.6
15			23.0	23.0
16			28.3	28.3
17			55.7	55.7
18	0.94 (3H, s)	0.94 (3H, s)	12.1	12.0
19	0.62 (3H, s)	0.62 (3H, s)	17.6	17.6
20			40.4	40.4
21	1.03 (3H, d, *J*= 6.4 Hz)	1.03 (3H, d, *J*= 6.4 Hz)	21.1	21.1
22	5.18 (1H, m)	5.14-5.25 (2H, m)	135.5	135.6
23	5.18 (1H, m)	5.14-5.25 (2H, m)	132.0	132.0
24			42.8	42.8
25			33.1	33.1
26	0.83 (3H, d, *J*= 6.4 Hz)	0.84 (3H, d, *J*=6.4 Hz)	19.6	19.6
27	0.81 (3H, d, *J*= 6.4 Hz)	0.82 (3H, d, *J*=6.4 Hz)	19.9	19.9
28	0.91 (3H, d, *J*= 6.8 Hz)	0.93 (3H, d, *J*= 6.8 Hz)	16.3	16.2

antitumor activity against Walker carcinosarcoma and human mammary adenocarcinoma cell lines in vitro (Jong and Donovik, 1989), as well as against human gastric tumor cell line (SNU-1), human hepatoma cell line (SNU-354), human colorectal tumor cell line (SNU-C4), and murine sarcoma-180 (Nam *et al.*, 2001). Studies showed that the cytotoxicity of ergosterol on HL60 cells results from its ability to induce apoptosis (Takei *et al.*, 2005). Ergosterol can be converted to several steroidal compounds with medicinal applications by both chemical and biochemical modifications (Fernandes *et al.*, 2003). Therefore, endophytic fungi can be used as biological synthesizer of sterol compounds like ergosterol.

Conclusion

This study is an attempt to explore the medicinal values of the endophytic fungi associated with the plant *P. niruri*. The study of plant associated endophytes could present a way of acquiring novel metabolites having diverse range of biological activities. From the two isolated endophytic fungi one strain, PNLE (*Fusarium* sp.) exhibited significant antifungal activity. Both the isolate exhibited significant cytotoxic activity in the brine shrimp lethality bioassay and mild antioxidant activity in the DPPH radical scavenging activity. The results indicate that the endophytic fungi from *P. niruri* with different bioactivities can be used as potential source of drug candidates. One sterol namely ergosterol has been isolated from the fungus PNRE (*Colletotrichum* sp.). The reports on the clinical uses of *P. niruri* have shown that the plant has a wide array of pharmacological activities. It can be concluded that *P. niruri* and its associated endophytic fungi has medicinal activity but it is time to explore its medicinal value at molecular level with the help of various techniques.

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