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RESEARCH ARTICLE

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Isolation and Cytotoxic Potency of Endophytic Fungi Associated with *Dysosma difformis*, a Study for the Novel Resources of Podophyllotoxin

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ABSTRACT

Endophytic fungi are promising sources for the production of podophyllotoxin-an important anticancer compound, replacing depleted medical plants. In this study, the endophytes associated with *Dysosma difformis*-an ethnomedicinal plant species were isolated to explore novel sources of podophyllotoxin. Fifty-three endophytic fungi were isolated and identified by morphological observation and ITS-based rDNA sequencing, assigning them to 27 genera in 3 divisions. *Fusarium* was found the most prevalent genus with a colonization frequency of 11.11%, followed by *Trametes* (9.26%) and *Penicillium* (7.41%). Phylogenetic trees were constructed for the endophytic fungi community in two collection sites, Ha Giang and Lai Chau, revealing the adaptation of the species to the specific tissues and habitats. Cytotoxic activity of endophytic fungal extracts was investigated on cancer cell lines such as SK-LU-1, HL-60, and HepG2, demonstrating strong anti-cancer activity of six isolates belonging to *Penicillium, Trametes, Purpureocillium, Aspergillus,* and *Ganoderma* with IC_{50} value of lower than 10 μg/mL. The presence of podophyllotoxin was indicated in *Penicillium, Trametes, Aspergillus* and for the first time in *Purpureocillium* and *Ganoderma via* high-performance liquid chromatography, which implied them as a potential source of this anti-cancer compound.

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1. Introduction

Endophytic fungi are fungal strains that colonize inside plant tissues without leading to any disease, instead, they are thought to enable their host to survive under biotic and/or biotic stress conditions or to improve growth [1–3]. Recently, endophytic fungi isolated from plants have been admitted as an important source of plant derived-raw materials for the pharmaceutical industry [3–5]. The production of plant-derived metabolites by endophytic fungal strains is supported by the three theories, namely, (1) horizontal gene transfer (HGT) event explained that during the long-term association of endophytes and host plants, endophytes adapt to the environment within plant tissues by genetic modification, including the integration of plant's genes into endophyte's genomes [6,7]; (2) xenohormesis hypothesis referred that endophytes experienced the same environmental signals as the host plant, hence, have acquired the ability to synthesize same or similar compounds [2,8] and (3) trait-specific endophytic infallibility presented endophytes, as independent organisms, gain plant-like-metabolism pathways as a result of evolutionary pressures [3,9]. This has possibly led to the ability of certain endophytic fungi to biosynthesize certain substances initially associated with host plants.

Podophyllotoxin (PTOX) is a broad spectrum bioactive metabolite, found in both plants and endophytic fungi. PTOX is cytotoxic and employed as the precursor in the biosynthesis of less toxic anticancer compounds such as teniposide, etoposide, etc. Previous studies have reported the successful production of podophyllotoxin from endophytic fungi isolated from PTOX producing plants such as *Phialocephala fortinii* from *Podophyllum peltatum* [10], *Fusarium* sp. (WB5121) from *D. versipellis* [11], *Fusarium solani* from *Podophyllum hexandrum* [12], *Aspergillus fumigatus* from *Juniperus communis* [13], *Alternaria tenuissima* from *Sinopodophyllum emodi* (Wall.) Ying [14], *Monilia* sp., *Penicillium implication* from *Dysosma veitchii* [15], providing the scientific evidence and showing the potential of endophytic fungi to replace the currently depleted resource

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of plants for PTOX production. Therefore, the study on the diversity of endophytes associated with medical plants could explore novel biological resources of PTOX with high social and economic value.

Dysosma difformis (hemsl & E.H. Wilson) T.H. Wang, a member of *Berberidaceae*, is an exceptional perennial herb that has been used as a folk remedy in Vietnam. Traditionally, they have been prevalently applied for the treatment of sore throats, pimples, snakebites, breast abscesses, and breast cancer [16]. Recently, PTOX was found in ethanol extract of *D. difformis* (Hemsl. & E.H. Wilson) T.H. Wang, which showed significant antioxidant and/or antidiabetic activity [16]. However, the diversity and bioactivity of endophytic fungi associated with *D. difformis* have not been reported yet.

In this study, we for the first time reported on the diversity of endophytic fungi isolated from *D. difformis* in Vietnam. Cytotoxicity of these fungi was assessed on three different cancer cell lines including SK-LU-1, HL-60, and HepG2 to look for potent PTOX-producing fungi. Further, the presence of PTOX in fungal extracts was identified using high-performance liquid chromatography (HPLC) method.

2. Materials and methods

2.1. Collection of plant materials

Healthy plants were collected randomly in two locations: (1) Phin Sang village, Minh Tan commune, Vi Xuyen district, Ha Giang province (23°0′40.14″N 104°53′6.92″E, altitude 1074 m) (2) Ngai Thau village, Khun Ha commune, Tam Duong district, Lai Chau province (22°13′2.55″N 103°35′0.58″E, altitude 1890 m). All samples were stored in sterile plastic bags at 4 °C.

2.2. Isolation of endophytic fungi

Samples were washed gently with tap water, then dried in a cool dry place before being used for isolation of fungal endophytes under sterile conditions in the fume hood Streamline Class II (ESCO, Troisdorf, Germany). Endophytic fungi were collected from D. *difformis* using methods described by Kusari et al. [9] with some modifications. The stems, roots, and leaves were washed with running tap water followed by distilled water, then cut into~1 cm pieces, dried on a sterile filter paper, and air-dried for 48 h at room temperature in a sterile inoculation box. Surface sterilization was then done by sequentially rinsing the plant materials with 70% ethanol for 3 min (for leaf samples) or 5 min (for root and stem samples), followed by sodium hypochlorite 5% (MERCK, Darmstadt, Germany) for 2 min (for leaf samples) or 3 min (for root and stem samples), soaked in 2.5% sodium thiosulfate solution (MERCK) for 5 min, then rinsed in 75% ethanol. After surface sterilization, the pieces were removed outer layer, ground, and spread on potato dextrose agar (PDA) plates containing 20% (w/v) potatoes, 2% D-glucose and 1.8% (w/v) agar. Each sample was repeated at least 3 times. The Petri dishes were incubated at 25 °C for 1–2 weeks until fungal mycelia started growing. These fungi were further subcultured on new PDA plates to obtain pure hyphae. The obtained fungal endophytes were coded according to location and original tissue (HG, LC encoding for samples from Ha Giang and Lai Chau, respectively; L, T, R encoding for samples from leaves, stems, and roots, respectively). These endophytes were stored at 25 °C.

2.3. Identification and phylogenetic evaluation of endophytic fungi

2.3.1. Morphological identification

Morphological identification of isolated endophytic fungi was conducted using characteristics such as surface texture, surface topography, and pigmentation, according to the taxonomy keys of Nguyen and Klich [17, 18].

2.3.2. Molecular identification

Endophytic fungi were identified by ITS-based rDNA sequencing. Each pure fungus was separately cultured in 100 mL PDA broth and shaken at 150 rpm at 25 °C for 3–7 days. Biomass of the endophytic fungi was collected by centrifugation at 6000 rpm for 10 min, and used for genomic DNA extraction with the G-spinTM Total DNA Extraction kit (INtRON Biotechnology, Sangdaewon, Korea) according to the manufacturer's protocol. The fungal ITS1-18S-ITS4 regions were amplified by PCR using primer pair ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4 5′-TCCTCCGCTTATTGATATGC-3′ in the reaction mixture containing 50 μL: 300 ng of purified PCR product, 1.5 μmol forward/reverse primer, Big Dye Terminator sequencing mix 25 μL (v. 3.1, Applied Biosystems, Foster, USA), following reaction program: 95 °C for 1 min, 36 cycles at 94 °C for 30 s, 53 °C for 20 s, 72 °C for 30 s, and additional extension at 72 °C for 5 min. The PCR products were checked for the expected size of 500–600 bp on 1% agarose gel. Sequencing of the samples was performed on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequenced fragments were analyzed by the program BioEdit sequence Alignment Editor software and blasted using the BLAST program against the NCBI GenBank database. The sequences were submitted to GenBank and accession numbers

were indicated in. Colonization frequency (CF,%) was calculated, using the following formula: $DF = \left(\frac{Ni}{Nt}\right)100$, in which Ni represents the number of segments colonized by genus i, Nt represents the total number of segments.

2.3.3. Phylogenetic analysis

All sequences were adjusted by eliminating gaps and missing data in Chromas 2.5 software before being used for phylogenetic analysis in MEGA7 software. The Neighbor-Joining method was used to infer the evolutionary history. The evolutionary distances were demonstrated using the Maximum Composite Likelihood method. Bootstrap values less than 50 were not shown on phylogeny [19].

2.4. Preparation of fungal extracts

Each endophytic fungi isolate was cultured in 100 mL of PDA broth and incubated for 5 days with mild shaking (150 rpm) at 25 ± 2 °C in dark. The culture was filtered through a clean filter paper to separate fresh biomass and culture filtrate. Fresh biomass was then washed gently with distilled water twice and dried at 45 °C–60 °C. Dry biomass was soaked in 100 mL methanol organic solvent for 24 h and then sonicated for 1 h repeated 3 times to obtain fungal biomass extracts. The fungal biomass extract and culture filtrate were mixed and then dried at 30 °C in the evaporator R300 (Buchi, Flawil, Switzerland) to collect the fungal extract for further studies.

2.5. Cytotoxicity of endophytic fungi

2.5.1. Human cancer cell lines and maintenance

HL-60 (Human acute leukemia), SK-LU-1 (Human lung carcinoma), and HepG2 (human hepatocellular carcinoma) cell lines were provided by Professor J. M. Pezzuto, Long Island University, US and Prof. Jeanette Maier, University of Milan, Italy. HL-60 and SK-LU-1 cells were grown in RPMI (Roswell Park Memorial Institute), HepG2 cells were cultured in DMEM (Dulbecco s Modified Eagle Medium) (Hyclone, Logan, UT, USA) containing 10% FBS (Fetal Bovine Serum), 100 µg/mL penicillin, 100 µg/mL streptomycin (Sigma, Roedermark, Germany). Cells were incubated at 37 °C in humidified conditions containing 5% CO_2. The cancer cells were treated with trypsin to separate cells, which were then counted and adjusted to the density of 10^5 cells. mL^{-1}.

2.5.2. Cytotoxicity assay

Cytotoxicity of fungal extracts was evaluated using sulforhodamine B (SRB) assay as described by Skehan et al. [20], with some modifications. Briefly, the cells were plated in a 96-well plate before fungal extracts at different concentrations (0.032; 0.16; 0.8; 4; 20; 100 µg/mL) or DMSO 1% (negative control), or Ellipticine (Sigma, Deisenhofen, Germany) at concentrations of 10 µg/mL; 2 µg/mL; 0.4 µg/mL; 0.08 µg/mL (positive control), or podophyllotoxin at concentrations of 0.032; 0.16; 0.8; 4; 20; 100 µg/mL were introduced into these wells. The wells containing only cancer cells were used as day 0 control and fixed with Trichloroacetic acid (TCA 20%)(Sigma, Deisenhofen, Germany) after one hour whilst other wells were incubated for 72 h before being fixed with TCA for 1 h. All wells were subsequently stained with SRB for 30 min at 37 °C, washed 3 times with acetic acid, and dried at room temperature. 10 mM unbuffered Tris base was added to dissolve the amount of SRB. Plates were gently shaken for 10 min and then read the OD results at 540 nm on an ELISA Plate Reader (Biotek, USA). The inhibition of cell growth was determined using the following formula: growth inhibition (%) = [100% – [(OD of sample – OD of day0)/(OD of negative control – OD of day 0)] × 100] [20].

The IC_{50} value (concentration that inhibition 50% of growth) was determined using Table Curve 2Dv4 computer software. The experiments were replicated one time with three biological replicates. IC_{50} values of fungal extracts, *D. differmis* extract and positive controls (podophyllotoxin and ellipticine) were assessed according to standards of the US National Cancer Institute (NCI) [21].

2.6. The detection of podophyllotoxin by high-performance liquid chromatography (HPLC)

Further confirmation of the PTOX in the fermentation broth was done by HPLC analysis using an Agilent 1200 HPLC system equipped with a diode array detector (DAD) (Agilent Technologies, Palo Alto, CA, USA), as described by Gupta et al. [22] with some modifications. Detection was performed in a range of 210 nm to 365 nm using DAD. The chromatograph acquisition was defined at 210 nm. The samples were separated on an Eclipse XDB-C18 reverse-phase column (250 mm × 4.6 mm, 5 µm particle size) (Agilent Technologies) at the room temperature (25.3–26.7 °C), with a mobile phase of H_2O (A)/acetonitrile (B) (Merck), the flow rate of 0.5 $mL.min^{-1}$, and 10 µL of injection volume. Gradient elution was used with linear increases from 10 to 20% B until 10 min, from 20 to 50% B until 23 min, from 50 to 90% B until 35 min, and isocratic elution of 90% B until 60 min. Fungal and plant extracts in methanol at a concentration of 10 mg/mL, and the standard PTOX (Sigma) in

methanol at a concentration of 1 mg/mL were used for the HPLC analysis. The identification of PTOX was conducted by comparison of retention times (Rt) and UV spectra with those of the standard PTOX [22].

3. Results

3.1. Isolation, identification and phylogenetic evaluation of endophytic fungi

Fifty-three endophytic fungi were isolated from *D. difformis*, including 28 isolates from samples in Ha Giang and 25 isolates from samples in Lai Chau. In Ha Giang, a large majority of endophytic fungi were isolated from roots with more than 80% (23 strains), followed by leaves with nearly 11% (3 strains) and stems with above 7% (2 strains). By contrast, in Lai Chau, only four strains were found in roots (14%) while the figures in leaves and stems were eleven and ten (44% and 40%), respectively. In total, the rate of isolation from roots is highest at 50.94%, followed by leaves and stems with 26.42% and 22.64%, respectively. All fifty-three isolates were identified by morphological observation (Figure S1, Table S1, Supplementary data) and ITS-based rDNA sequencing. In general, the isolates belonged to twenty-seven genera, of which most isolates were Ascomycetous fungi, along with three Basidiomycetous fungi, a Mucormycetous fungus, and an unidentified fungus. The colonization frequency of each isolated fungus was calculated, showing *Fusarium* is the most prevalent genus (11.11%), followed by *Trametes* (9.26%) and *Penicillium* (7.41%) (Table 1).

Further, two phylogenetic trees describing the taxonomic relationship between the endophytic fungi in two sites of collection were also constructed using MEGA 7 software. Bootstrap values less than 50 were not shown on phylogeny. In Ha Giang, endophytic fungi were constructed into two clusters, of which cluster 1 included genera of Ascomycota Division, and cluster 2 consisted of genera of Mucoromycota and Basidiomycota Division. Noticeably, *Trametes*-a Basidiomycetous fungus was located in the same sub-clade with *Mucor* sp., which separated to *Apiotrichum*. But bootstrap values were not confident (less than 50). Interestingly, *Fusarium* strains isolated in leaves and roots were located in different branches, which could be hypothesized because of the adaptation of endophytes to different tissues (Figure S2, Supplementary data). In comparison, almost genera of the Ascomycota Division were located in cluster 1 with *Mucor* while Basidiomycetous genera were located in cluster 2 on phylogeny constructed by endophytes collected in Lai Chau. Unique LCN12.1L identified as *Penicillium herquei* 50SG10-an Ascomycetous fungus was situated in cluster 3 (Figure S3, Supplementary data). The difference in the structure of phylogeny between the two sites of collection suggested the different habitats possibly influence the genetic evolution and diversity of endophytes inhabiting *D. difformis*.

3.2. Cytotoxicity of endophytic fungi extracts

The *in vitro* cytotoxic activities of fungal extracts were investigated on three cancer cell lines (SK-LU-1, HL 60, and Hep2) in comparison to *D. difformis* extract and positive controls such as podophyllotoxin and ellipticine. IC_{50} values of these extracts were evaluated using at least four different concentrations. According to the standards of the US National Cancer Institute (NCI), $IC_{50}<20\ \mu g/mL$ for extracts and $IC_{50}<5\ \mu g/mL$ for the purified substance were considered good activities [21,23–25]. The results revealed twenty-eight extracts displayed cytotoxic activity on SK-LU-1 cells with IC_{50} from $0.036 - 80.23\ \mu g/mL$, of which twenty-one extracts showed strong cytotoxicity on SK-LU-1 cells with IC_{50} from 0.036 to 18.75 µg/mL (Table 2).

Twenty-eight extracts showing cytotoxic activity against SK-LU-1 were further investigated for anticancer activity against HL-60 (Human acute leukemia) and HepG2 (human hepatocellular carcinoma). Six extracts belonging to *Penicillium* (two isolates), *Trametes, Purpureocillium, Aspergillus,* and *Ganoderma* genera showed very strong cytotoxic activity against HL-60 and HepG2 with IC_{50} from 0.073 to 0.31 µg/mL and from 1.60 to 10.84 µg/mL, respectively (Table 3) while others did not show any cytotoxic activity (data not shown).

3.3. The detection of podophyllotoxin in endophytic fungi extracts by high-performance liquid chromatography (HPLC)

HPLC analysis was described as the accurate, and precise tool for the determination of PTOX in plant extracts and fungal extracts [26–29]. Six extracts found to be active against three cancer cell lines were tested for the presence of podophyllotoxin using HPLC analysis. The retention time (Rt) developed by the standard PTOX (Sigma) was analyzed. In the first HPLC assay, the chromatographic peaks of HGN13R, HGN12.2R, LCN3T, and LCN8T extracts with the respective Rt at 37.164, 37,209, 37.197, and 37.202 min were similar to that of the standard (Rt = 37.130 min). In the second HPLC assay, the chromatograms of HGN12.1R, HGN13.1R, and plant extracts showed the peaks with Rt at 37.359, 37.378, and 37.353 min, respectively, which were also corresponding to that of the standard PTOX (Table 4). Clear UV spectra were

Table 1. Endophytic fungi were isolated from *Dysosma difformis*.

No	Fungal isolate	Location	Plant tissue	Accession number	Closest relatives in NCBI	ITS identity (%)	Genera, Division	Colonization frequency of genera (CF, %)
1	HGN6.1R	Ha Giang	Root	MZ461976	*Apiotrichum dehoogii* culture CBS:8686 (KY101656.1)	99.80	*Apiotrichum*, Basidiomycota	1.85
2	HGN6.2R	Ha Giang	Root	MZ468130	*Aspergillus* sp. 151304 (MT371256.1)	91.94	*Aspergillus*, Ascomycota	5.56
3	HGN13R	Ha Giang	Root	MZ462046	*Aspergillus flavus* GFRS9 (MT447477.1)	100		
4	LCN9R	Lai Chau	Root	MZ470737	*Aspergillus nomius* JIXX-FS31 (KR296870.1)	99.82		
5	LCN21T	Lai Chau	Stem	MZ470733	*Cercospora citrullina* C2015 (KY593165.1)	100	*Cercospora*, Ascomycota	1.85
6	HGN1R	Ha Giang	Root	ON142327	*Cladosporium sphaerospermum* SCAU177 (MH464425.1)	99.82	*Cladosporium*, Ascomycota	3.70
7	LCN7L	Lai Chau	Leaf	MZ470614	*Cladosporium* sp. XHMEA3 (MH985344.1)	100		
8	HGN8R	Ha Giang	Root	MZ469934	*Clonostachys rogersoniana* voucher IFRD500-015 (MT559105.1)	100	*Clonostachys*, Ascomycota	3.70
9	LCN1R	Lai Chau	Root	MZ477226	*Clonostachys rosea* 197 WS (MG396999.1)	100		
10	HGN11.1R	Ha Giang	Root	MZ461968	*Colletotrichum gloeosporioides* Y35 (MT729915.1)	100	*Colletotrichum*, Ascomycota	5.56
11	LCN3L	Lai Chau	Leaf	MZ470466	*Colletotrichum kahawae* WZ-135 (MN856281.1)	100		
12	LCN19T	Lai Chau	Stem	MZ470465	*Colletotrichum acutatum* WZ-298 (MN856415.1)	99.27		
13	LCN8.3L	Lai Chau	Leaf	MZ470419			*Fungal* sp.	1.85
14	HGN7.1R	Ha Giang	Root	MZ461980	*Fusarium oxysporum*_LZ070103 (FJ157216.1)	99.80	*Fusarium*, Ascomycota	11.11
15	HGN7R	Ha Giang	Root	MZ461979	*Fusarium oxysporum* LZ070103 (FJ157216.1)	99.6		
16	HGN5L	Ha Giang	Leaf	MZ461981	*Fusarium solani* LJ02 (MT579863.1)	99.81		
17	LCN1L	Lai Chau	Leaf	MZ470447	*Fusarium* sp. 2 JM-2014 (KJ920732.1)	88.71		
18	LCN6T	Lai Chau	Stem	MZ472087	*Fusarium* sp. 2 JM-2014 (KJ920732.1)	88.74		
19	LCN1T	Lai Chau	Stem	MZ470445	*Fusarium* sp. 2 JM-2014 (KJ920732.1)	92.48		
20	LCN8T	Lai Chau	Stem	MZ470735	*Ganoderma lingzhi* AL-R5 (MH160076.1)	100	*Ganoderma*, Basidiomycota	1.85
21	HGN2R	Ha Giang	Root	MZ461975	*Guignardia* sp. voucher HQU PL08 (MK640597.1)	99.83	*Guignardia*, Ascomycota	1.85
22	HGN3R	Ha Giang	Root	MZ461966	*Ilyonectria destructans* (MK403674.1)	100	*Ilyonectria*, Ascomycota	5.56
23	HGN7.2R	Ha Giang	Root	MZ461985	*Ilyonectria robusta* JJHHD-6 (MN091953.1)	99		
24	LCN16L	Lai Chau	Stem	MZ470426	*Ilyonectria* sp. HB 5 (KP761761.1)	99.80		
25	HGN14.1R	Ha Giang	Root	MZ462039	*Lasiodiplodia venezuelensis* CBS 129753 (MH865369.1)	100	*Lasiodiplodia*, Ascomycota	1.85
26	HGN5R	Ha Giang	Root	MZ469936	*Mucor* sp. RL288 (MT557384.1)	99.29	*Mucor*, Mucoromycota	3.70
27	LCN7R	Lai Chau	Root	MZ470734	*Mucor circinelloides* M (MK791718.1)	99.47		
28	HGN6R	Ha Giang	Root	MZ461978	*Neurospora intermedia* WS11JB14 (KT844672.1)	99.45	*Neurospra*, Ascomycota	1.85
29	HGN13.1R	Ha Giang	Root	MZ468131	*Penicillium citrinum* JBGC3.4 (MG554246.1)	100	*Penicillium*, Ascomycota	7.41
30	HGN12.1R	Ha Giang	Root	MZ462047	*Penicillium herquei* BCC (THA):84323 (MF537646.1)	99.80		
31	LCN15L	Lai Chau	Leaf	MZ470422	*Penicillium herquei* LTL319 (MF663569.1)	99.81		
32	LCN12.1L	Lai Chau	Leaf	MZ470423	*Penicillium herquei* 50SG10 (MH986808.1)	100		
33	HGN1T	Ha Giang	Stem	MZ461977	*Pestalotiopsis* sp. SC24c (MG489866.1)	99.80	*Pestalotiopsis*, Ascomycota	1.85
34	LCN12T	Lai Chau	Stem	MZ470448	*Phaeoacremonium* sp. MRHf10 (MK120896.1)	100	*Phaeoacremonium*, Ascomycota	1.85
35	HGN14R	Ha Giang	Root	MZ461983	*Phaeosphaeriopsis musae* A731 (KU529845.1)	100	*Phaeosphaeriopsis*, Ascomycota	3.7
36	HGN3L	Ha Giang	Leaf	MZ461982	*Phaeosphaeriopsis musae* A731 (KU529845.1)	100		
37	HGN1L	Ha Giang	Leaf	MZ461986	*Phyllosticta capitalensis* H (ON014497.1)	99.49	*Phyllosticta*, Ascomycota	3.70
38	LCN5L	Lai Chau	Leaf	MZ470604	*Phyllosticta capitalensis* PN-2014-08-28-A1 (KT319044.1)	99.83		

(*continued*)

Table 1. Continued.

No	Fungal isolate	Location	Plant tissue	Accession number	Closest relatives in NCBI	ITS identity (%)	Genera, Division	Colonization frequency of genera (CF, %)
39	HGN3T	Ha Giang	Stem	MZ462053	*Plectosphaerella oligotrophica* CGMCC 3.15078 (NR_155632.1)	99.42	*Plectosphaerella*, Ascomycota	3.70
40	LCN14L	Lai Chau	Leaf	MZ470459	*Plectosphaerella cucumerina* PCO.52 (HQ248206.1)	99.60		
41	LCN3T	Lai Chau	Stem	MZ470446	*Purpureocillium lilacinum* PIYN7703 (MH483756.1)	99.53	*Purpureocillium*, Ascomycota	1.85
42	LCN13T	Lai Chau	Stem	MZ470584	*Setophoma* sp. PSRF58 (MN888811.1)	100	*Setophoma*, Ascomycota	1.85
43	LCN17T	Lai Chau	Stem	MZ470424	*Simplicillium* sp. FTJZZJ01 (FJ196609.1)	99.81	*Simplicillium*, Ascomycota	1.85
44	LCN4T	Lai Chau	Stem	MZ471623	*Talaromyces amestolkiae* CBS 274.95 (KP975419.1)	100	*Talaromyces*, Ascomycota	1.85
45	HGN11R	Ha Giang	Root	MZ469933	*Trametes* cf. *cubensis* 11B-25 (KJ654514.1)	99.83	*Trametes*, Basidiomycota	9.26
46	HGN12.2R	Ha Giang	Root	MZ461967	*Trametes* cf. *cubensis* 11B-25 (KJ654514.1)	100		
47	HGN3.1R	Ha Giang	Root	MZ461984	*Trametes* cf. *cubensis* 11B-25 (KJ654514.1)	100		
48	LCN11L	Lai Chau	Leaf	MZ470263	*Trametes* cf. *cubensis* 11B-25 (KJ654514.1)	99.83		
49	LCN13L	Lai Chau	Leaf	MZ470264	*Trametes* cf. *cubensis* 11B-25 (KJ654514.1)	99.5		
50	HGN2.1R	Ha Giang	Root	MZ469935	*Trichoderma citrinoviride* SC2e (MG461650.1)	97.01	*Trichoderma*, Ascomycota	5.56
51	HGN5.1R	Ha Giang	Root	MZ462050	*Trichoderma citrinoviride* SC2e (MG461650.1)	97.01		
52	LCN3R	Lai Chau	Root	MZ477227	*Trichoderma lixii* NG5 (KX580184.1)	99.82		
53	HGN10R	Ha Giang	Root	MZ469932	*Xylaria* sp. NBRTSLF-46 (MF807974.1)	99.24	*Xylaria*, Ascomycota	1.85

obtained for the HGN13R, HGN12.2R, LCN3T, and LCN8T extracts, revealing that could separate them from other types of compounds. In addition, the comparison of the UV spectra of the aforementioned HPLC chromatographic peaks with that of the standard PTOX revealed an excellent match, confirming the presence of PTOX in fungal extract (Figure 1, Figure S4 (Supplementary data)). The UV spectra of HGN12.1R, HGN13.1R, and plant extracts exhibited a similar pattern to that of the standard PTOX though they were relatively inconspicuous.

4. Discussion

For past decades, endophytic fungi are considered a promising resource of bioactive compounds, especially plant-derived therapeutic drugs because of their capability for producing host-like compounds. Therefore, investigating the diversity of medical plant-associated fungi is an excellent approach for the exploration of novel microbial manufacturers producing medicinally useful compounds. In the previous study, *D. differmis* was reported for the production of podophyllotoxin [16], suggesting endophytic fungi harboring this herb could synthesize PTOX. The present study is the first report on the diversity and bioactivity of endophytic fungi inhabiting *D. differmis*, for exploring novel PTOX-producing endophytic fungi.

The results of isolation and identification indicated for the first time two genera *Apiochitrum* and *Ganoderma* of Basidiomycota were found on the herbaceous plants. Noticeably, we assigned *Mucor* sp. into Mucormycota instead of Zygomycota as described by Tan et al., (2018) because the most recent taxonomy allocated Zygomycetous fungi into Zoopagomycota, Mucoromycota, and Glomeromycota [30]. The phylogenetic analysis revealed the adaptation of the species to the specific tissues and habitats, which is consistent with the conclusion of Gupta et al., who reviewed that site of collection, sample size (size of explants), and localization of fungal endophytes in plant tissues can influence the endophytic fungal community [31].

Cytotoxicity assay revealed strong activity of six isolates including *Penicillium* sp. (HGN 12.1 R), *Penicillium* sp. (HGN 13.1 R), *Trametes* sp., *Purpureocillium* sp., *Aspergillus* sp., and *Ganoderma* sp. against all of three cancer cell lines, namely SK-LU-1, HL-60, and HepG2, which have never seen in previous studies on the anticancer activity of endophytic fungi. For example, on HL-60 cells, cyclo (L-[4-hydroxyprolinyl]-L-leucine), cyclo (L-Phe-trans-4-hydroxy-L-Pro), and phenethyl acetate isolated from *Streptomyces griseus* showed antiproliferative activity with IC_{50} values of 115.7, >200,

Table 2. Cytotoxicity IC_{50} values of fungal extracts on SK-LU-1 cells.

No	Isolate code	Cytotoxic activity IC_{50} (μg/mL)	No	Isolate code	Cytotoxic activity IC_{50} (μg/mL)
1	HGN6.1R	>100	29	HGN13.1R	4.030 ± 0.600
2	HGN6.2R	80.230 ± 2.420	30	HGN12.1R	1.190 ± 0.110
3	HGN13R	0.036 ± 0.001	31	LCN15L	80.220 ± 1.740
4	LCN9R	0.163 ± 0.012	32	LCN12.1L	0.195 ± 0.027
5	LCN21T	1.770 ± 0.150	33	HGN1T	>100
6	HGN1R	75.29 ± 2.580	34	LCN12T	2.230 ± 0.290
7	LCN7L	6.440 ± 0.410	35	HGN14.1R	>100
8	HGN8.1R	3.030 ± 0.300	36	HGN3L	>100
9	LCN1R	>100	37	HGN1L	>100
10	HGN11.1R	>100	38	LCN5L	>100
11	LCN3L	>100	39	HGN3T	>100
12	LCN19T	5.580 ± 0.450	40	LCN14L	>100
13	LCN8.3L	>100	41	LCN3T	2.040 ± 0.190
14	HGN7.1R	>100	42	LCN13T	28.660 ± 1.500
15	HGN7R	>100	43	LCN17T	3.900 ± 0.260
16	HGN5L	>100	44	LCN4T	>100
17	LCN1L	1.370 ± 0.140	45	HGN11R	63.320 ± 6.410
18	LCN6T	>100	46	HGN12.2R	2.060 ± 0.310
19	LCN1T	18.750 ± 1.110	47	HGN3.1R	9.590 ± 0.150
20	LCN8T	2.490 ± 0.220	48	LCN11L	0.110 ± 0.036
21	HGN2.1R	0.550 ± 0.050	49	LCN13L	>100
22	HGN3R	>100	50	HGN2R	0.069 ± 0.004
23	HGN7.2R	>100	51	HGN5.1R	76.670 ± 2.680
24	LCN16T	>100	52	LCN3R	>100
25	HGN14R	>100	53	HGN10R	72.010 ± 6.430
26	HGN5R	>100	54	*D. difformis* extract	0.160 ± 0.020
27	LCN7R	>100	55	Podophyllotoxin	0.054 ± 0.006
28	HGN6R	2.090 ± 0.120	56	Ellipticine[a]	0.430 ± 0.040

[a]Podophyllotoxin, Ellipticine were used as positive controls.

Table 3. Cytotoxicity IC_{50} values of fungal extracts on HL-60 and HepG2 cells.

			Cytotoxic activity IC_{50} (μg/mL)	
Isolate number	Isolate code	Isolate name	HL-60	HepG2
1	HGN12.1R	*Penicillium*	0.013 ± 0.004	0.071 ± 0.011
2	HGN12.2R	*Trametes*	0.056 ± 0.009	0.200 ± 0.030
3	HGN13R	*Aspergillus*	0.310 ± 0.030	1.600 ± 0.150
4	HGN13.1R	*Penicillium*	0.073 ± 0.006	10.840 ± 1.190
5	LCN3T	*Purpureocillium*	0.028 ± 0.005	0.013 ± 0.003
6	LCN8T	*Ganoderma*	0.046 ± 0.007	2.020 ± 0.160
7	*D. difformis* extract		0.840 ± 0.0800	1.110 ± 0.150
8	Podophyllotoxin		0.008 ± 0.001	0.180 ± 0.020
9	Ellipticine[a]		0.280 ± 0.030	0.370 ± 0.040

[a]Podophyllotoxin, Ellipticine were used as positive controls.

Table 4. HPLC data for the identification of PTOX.

Analytes	Retention time (Rt, min)
Podophyllotoxin	37.130
HGN13R	37.164
HGN12.2R	37.209
LCN3T	37.197
LCN8T	37.202
Podophyllotoxin	37.365
HGN12.1R	37.359
HGN13.1R	37.378
Plant extract (*D. difformis* extract)	37.353

HPLC analyses were performed two times with two separated standard podophyllotoxin. PTOX in the extracts of HGN13R, HGN12.2R, LCN3T, LCN8T was identified using the corresponding podophyllotoxin; PTOX in the extracts of HGN13.1R, HGN12.1R was identified using the corresponding podophyllotoxin.

and 74.7 μg/mL [32] while the CH_2Cl_2 extract of *Artemisia turanica* showed the same effect with IC_{50} values of 104 μg/mL [33]. In another study, the methanol extract of Chaga mushroom *Inonotus obliquus* exhibited moderate cytotoxicity against HL-60, LU-1, SW480, and HepG2 with values of 32.2, 38.0 μg/mL, 41.3 μg/mL, and 51.3 μg/mL, respectively [34]. Rahaman et al. [35] reported cytotoxic activity of eighty fungal endophytes isolated from Sundarbans mangrove plants on MCF-7 and SK-LU-1 cells, which revealed solely *Talaromyces harzianum* (HFSF-1) has good cytotoxic activity on both cell lines (IC_{50} < 20 μg/mL). *Talaromyces* sp and *Aspergillus oryzae* demonstrated a significant toxic effect on MCF-7 cells (IC_{50} < 20 μg/mL) only while *Penicillium chrysogenum* showed just good activity on the SK-LU-1 cells [35].

The results from cytotoxic assays implied six isolates could produce podophyllotoxin. Indeed, chromatogram and UV spectra profiling from HPLC analysis confirmed the presence of PTOX in all six fungal extracts. UV spectra of HGN12.1R, HGN13.1R, and plant extract were inconspicuous, which is possibly the result of the complexity of metabolites or the very low concentration of PTOX in these extracts. In the previous study, *Aspergillus*, *Penicillium*, *Trametes* were demonstrated as the

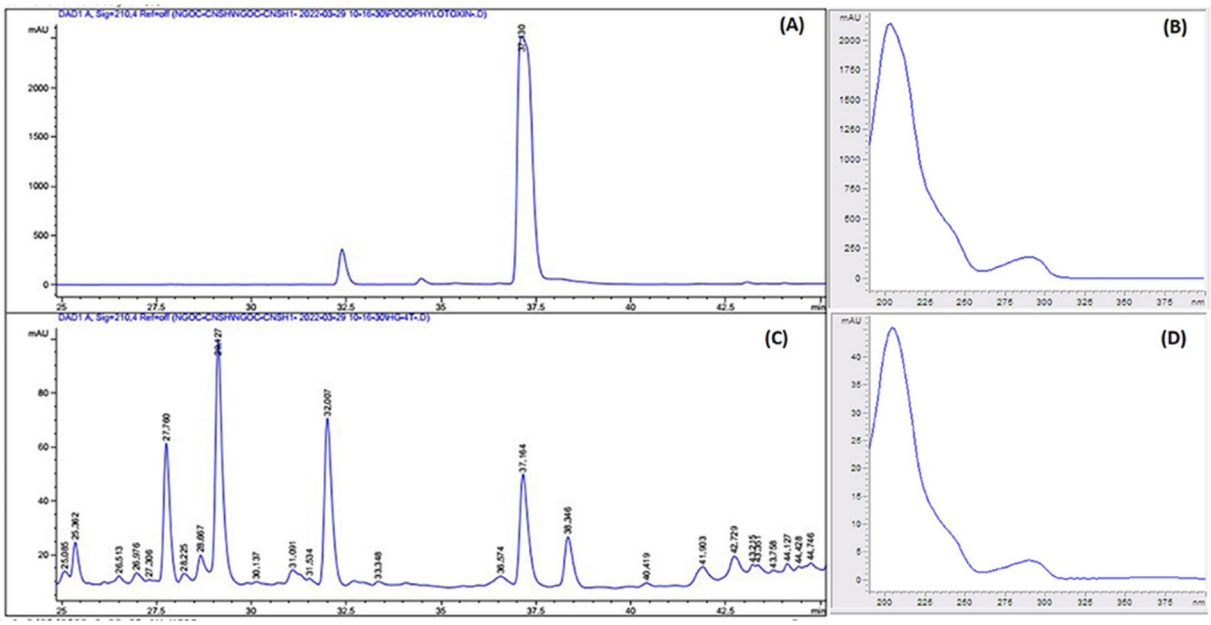

Figure 1. Identification of PTOX by HPLC-DAD. Chromatogram (A) and UV spectrum (B) of the standard PTOX (Sigma); Chromatogram (C) and UV spectrum (D) of HGN13R extract.

reservoirs of PTOX [36]. Particularly, *Penicillium implication* from *Dysosma veitchii* was found for producing PTOX [15]. Puri et al. isolated and characterized *Trametes hirsuta* that produces PTOX as shown by HPLC and LC-MS [37]. Surprisingly, for the first time, the presence of PTOX was discovered in *Ganoderma* sp, which is possibly a result of cohabitation with *D. difformis*. *Ganoderma lucidum* (Lizing) is a well-known medical mushroom that has been used in the treatment of various diseases such as cancer and diabetes [38,39]. The cultivation of *G. lucidum* was conducted by solid-state fermentation and sub-liquid state fermentation for the production of biomass and important compounds like extracellular polysaccharides [39]. Our finding revealed *G. lucidum* could be applied for the production of PTOX. In the previous study by Lenta et al., *Purpureocillium lilacinum* isolated from the roots of *Rauvolfia macrophylla* was well investigated on bioactivity and chemical components [40], but no PTOX was found in this species. The present study is the first report showing the production of PTOX in *P. lilacinum*. Taken together, these results strongly supported the hypothesis that endophytes could produce host-derived compounds. In further studies, the quantification of podophyllotoxin and genes involved in the biosynthesis of PTOX in endophytic fungi would be investigated, which could be mined for the large-scale production of this compound.

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Disclosure statement

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