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RESEARCH ON ISOLATION OF ENDOPHYTIC FUNGI FROM BAT GIAC LIEN MEDICINAL PLANT AND EVALUATION OF THE ABILITY TO PRODUCE THE ACTIVE COMPOUND PODOPHYLLOTOXIN AND ITS DERIVATIVES

SUMMARY OF APPLIED BIOLOGY PH.D. THESIS

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INTRODUCTION

1. Rationale for the study

Podophyllotoxin, an aryltetralin lignan, is a vital source for important anticancer drugs such as etoposide, teniposide, and etophophos. Podophyllotoxin is currently obtained mainly from certain plant species of the genus *Podophyllum* (*Podophyllum hexandrum*, *Podophyllum peltatum*), Tao ma tree (*Podophyllum peltatum*) where the major component is podophylloxin, or from certain species of the genus *Juniperus* (*Juniperus sabina, Juniperus thurifera, Juniperus chinensis*...), and some species of the genus *Linum* (Xiang et al., 2017). Hiện nay, rễ /thân rễ của *Podophyllum hexandrum* tạo thành một trong những nguồn quan trọng nhất của podophyllotoxin. Currently, the roots/rhizomes of *Podophyllum hexandrum* constitute one of the most important natural sources of podophyllotoxin. The chemical synthesis of podophyllotoxin is considered highly complex and inefficient due to the presence of four chiral centers along with a C lactone and a high oxidation level. Furthermore, the use of plant cell biomass culture and plant organ culture technologies in species capable of producing podophyllotoxin is difficult to implement due to the low yield of recovered podophyllotoxin.

Recently, endophytic fungi isolated from plants belonging to the genera *Podophyllum* (*Dysosma*) or *Juniperus* have been proven to be capable of producing podophyllotoxin. Successful research on the isolation of endophytic fungal strains, including *Fusarium oxysporum* from *Juniperus recurve*; *Penicillium sp*., *Phialocephala fortinii, Trametes hirsuta, Alternaria neesex* from *Podophyllum hexandrum*; *Monilia sp., Penicillium implication* from *Dysosma veitchii* in the production of podophyllotoxin demonstrates a scientific basis and the potential to replace the current dwindling raw material source. In Vietnam, species belonging to the genus *Podophyllum* include *Podophyllum tonkinense* Gagnep, 1938 – Bát giác liên and *Podophyllum versipelle* Hance, 1883, primarily distributed in northern

provinces such as Lai Châu, Lào Cai, Yên Bái, Hà Giang, and Lạng Sơn. Additionally, two introduced species, *Juniperus chinensis* (Chinese juniper) and *Juniperus squamata* (Scaly juniper), are present in Vietnam (Nguyễn Tiến Bân, 2003). These species have been proven by researchers both domestically and internationally to have the capacity to synthesize podophyllotoxin and deoxypodophyllotoxin, and are potential plant raw materials for the isolation of endophytic fungi (microorganisms) capable of producing podophyllotoxin and deoxypodophyllotoxin, thereby replacing the currently depleting natural source.

Based on that, we conducted the thesis "***Study on the isolation of endophytic fungi from the medicinal plant Bat giac lien and evaluation of their potential to produce podophyllotoxin and its derivatives***".

2. Research objectives:

To isolate and select endophytic fungal strains from the Bat giac lien plant that are capable of biosynthesizing podophyllotoxin and its bioactive derivatives. To sequence the genome of the selected fungal strains. To determine the appropriate fermentation conditions for the biosynthesis of podophyllotoxin and its derivatives. To purify and identify the chemical structure of podophyllotoxin/podophyllotoxin precursors from the selected endophytic fungi.

3. Research contents:

- Isolation, identification, and screening of endophytic fungal strains producing podophyllotoxin
- Decode the genome of the selected endophytic fungal strain and propose the biosynthetic pathway of podophyllotoxin or its precursors.
- Study of appropriate conditions for the biosynthesis of podophyllotoxin and its derivatives by the selected endophytic fungal strain using fermentation technology.
- Fermentation, recovery, determine the structure and evaluate the biological activity of the compounds from selected endophytic fungal strains.

CHAPTER I. RESEARCH OVERVIEW

The Bat giac lien plant belonging to the genus *Podophyllum* (*Dysosma*). This medicinal species was collected in northern provinces such as Lai Châu, Lào Cai, Yên Bái, Hà Giang, and Lạng Sơn. Within the scope of this thesis, Bat giac lien samples were collected in Hà Giang and Lai Châu. According to the Vietnamese Medicinal Plant Dictionary and the Red Book of Vietnam, "its roots and rhizomes contain berberine, and are traditionally used to treat acne, itching, eczema, sore throat, contusions/bruises, cuts, detoxification, indigestion, antiseptic, and venomous snake bites". This demonstrates the potential of the Bat giac lien plant as a source of valuable and rare genetic resources.

Previous studies have shown that podophyllotoxin (PTOX) is a well-known chemical compound found among hundreds of substances isolated from plants, primarily from the roots and stems of a species belonging to the genus *Podophyllum* (or *Dysosma*). Several domestic studies have resulted in the extraction and isolation of constituents from the underground parts of the Bat giac lien plant (*Podophyllum tonkinense* Gagnep.). The results showed the isolation of seven compounds from the underground parts (roots and rhizomes) of the Bat giac lien plant collected in Sa Pa (Lào Cai), which were identified as quercetin (1), rutin (2), kaempferol (3), nicotiflorin (4), α-peltatin (5), deoxypodophyllotoxin (6), and podophyllotoxin (7). Thus, the potential for exploiting valuable compounds from this medicinal species is significant and currently garnering much interest. This direction guides the research of genes involved in the metabolic pathways for the biosynthesis of therapeutic compounds, as well as methods to enhance the production and

storage of these compounds in order to conserve and replace the currently rare raw material source.

However, these plant resources are now facing the risk of decline due to over-exploitation. Therefore, a safe and promising approach to obtaining this valuable genetic resource without causing ecological imbalance is through isolation of endophytic fungi from this medicinal species. In addition to its potential as a natural anti-cancer compound, PTOX also possesses various other therapeutic properties found in several species of the genus *Podophyllum*, such as *P. hexandrum*, *P. peltatum*, *P. emodi*, v.v. Nó không chỉ giới hạn ở chi *Podophyllum* mà còn được tìm thấy ở một số chi khác chẳng hạn như *Jeffersonia, Diphylleia* và *Dysosma* (*Berberidaceae*), *Catharanthus* (*Apocynaceae*), *Polygala* (*Polygalaceae*), *Anthriscus* (*Apiaceae*), *Linum* (*Linaceae*), *Hyptis* (*Verbenaceae*), *Teucrium, Nepetaand Thymus* (*Labiaceae*), *Thuja*, *Juniperus*, *Callitrisand Thujopsis* (*Cupressaceae*), *Cassia* (*Fabaceae*), *Haplophyllum* (*Rutaceae*), *Commiphora* (*Burseraceae*), *Hernandia.* Numerous studies worldwide have also focused on podophyllotoxin and its derivatives, which are known to be important secondary metabolites of plants with significant medicinal value. They exhibit various bioactivities, such as PTOX's ability to induce cytotoxicity and function as a microtubule assembly inhibitor. This compound has been proven effective against lung cancer, testicular tumors, neuroblastoma, liver cancer, and other tumors. Podophyllotoxin is also known for its antiviral, antioxidant, antibacterial, immunostimulatory, and antirheumatic properties, primarily occurring in species of the genera *Juniperus* and *Podophyllum*. Furthermore, PTOX also has an endogenous origin (is produced endogenously).

Endophytic fungi are defined as fungi that spend all or part of their life cycle residing within the healthy tissues of a host plant, in various organs such as the stem, root, and branch—typically without causing obvious disease symptoms. The root zone is the point of entry for many invading endophytic microorganisms. Once they colonize the host, these fungi can accumulate at the invasion site or migrate throughout the plant, reaching the vascular systems of the root, stem, and leaves. They can promote various metabolic processes in the plant, significantly enhance root hair development, and increase root longevity. Throughout long evolutionary periods, some endophytic microorganisms co-existing with their host plants have established a special mutualistic relationship that can significantly influence the formation of various plant secondary metabolites. This underscores the relevance of studying the relationships between endophytes and their hosts, this is a novel/new approach for the efficient and substitutable production of scarce and valuable bioactive compounds.

CHAPTER 2. SUBJECTS AND RESEARCH METHODOLOGY

2.1. RESEARCH SUBJECTS

- Plant Samples: Healthy medicinal plant samples of *Dysosma differmis* (hemsl.&E.H.Wilson) T.H.Wang were randomly collected in Ha Giang and Lai Chau provinces.
- Bacterial Strains Used: Gram-negative bacteria (*Escherichia coli* DH5) and Gram-positive bacteria (*Staphylococcus* sp., *Bacillus* 7TM11, *Bacillus* 2TM6, *Enterobacter* 14RM7-1) were preserved/maintained at the Plant Cell Technology Laboratory, Institute of Biology, VAST.
- Human Cancer Cell Lines: SK-LU-1 (lung cancer cells); HepG2 (liver cancer cells); HL-60 (acute promyelocytic leukemia cells) provided by Prof. Dr. J. M. Pezzuto, Long-Island University, US, and Prof. Jeanette Maier, University of Milan, Italy.

2.2. RESEARCH METHODOLOGY

2.2.1. Method of sample collection and identification of Bat giac lien

The *Bat giac lien* samples were collected and classified by experts in ecology and taxonomy, including Trinh Ngoc Bon from the Vietnam Forest Science Institute and Nguyen Thi Thanh Huong from the Institute of Biology, Vietnam Academy of Science and Technology.

2.2.2. Method of plant extraction from *Bat giac lien root sample*

The Bat giac lien samples were thoroughly washed multiple times with distilled water, surface sterilized with 70% ethanol for 5 minutes, and then cut into small 1 cm pieces under aseptic conditions. They were then crushed and oven-dried until a constant weight was achieved. 100 g of the dried powder were soaked in 100 ml of methanol for 24 hours and subjected to ultrasonication for 1 hour. This process was repeated 3 times to obtain the crude extract solution. Subsequently, the extract solution was evaporated using a rotary evaporator (model R300) at 30 °C to obtain the dry crude extract for subsequent studies.

2.2.3. Method for isolation and identification of endophytic fungi

Surface sterilization protocol

The surface sterilization was performed by treating the samples with 70% ethanol for 3 minutes (for leaf samples) or 5 minutes (for root and stem samples), followed by rinsing with 5% sodium hypochlorite for 2 minutes (for leaf samples) or 3 minutes (for root and stem samples). The samples were then soaked in 2.5% sodium thiosulfate solution for 5 minutes, and subsequently rinsed with 75% ethanol. The samples were briefly washed several times with sterile distilled water, dried using sterile filter paper, and finally placed on LB agar plates. After 5 days at 25 °C, samples that showed no signs of bacterial or fungal growth around the plant tissue were confirmed to be clean and suitable for the next step of endophytic fungi isolation.

Isolation of Endophytic Fungi

Step 1: The surface-sterilized stem, leaf, and root samples were crushed using a sterile mortar and pestle.

Step 2: The crushed samples were streaked onto PDA plates. Each sample was streaked at least 3 times and incubated at 25±2 °C for 1−2 weeks. Fungal colonies that grew from the crushed plant tissues were considered endophytic fungi. These fungi were then purified onto new plates to obtain pure fungal cultures.

Fungal identification using traditional and molecular methods

- Initially, the fungal isolates were preliminary identified based on traditional methods, including characteristics such as colony color, morphology, size, shape, colony margin, mycelial color, under-surface color, exudate droplets, diffusible pigment, septate hyphae, spores, and spore stalk features for the preliminary identification and classification of fungal strains up to the genus level.

- Fungal DNA was extracted and subjected to PCR amplification to clone the target genes using the following specific primers:

  - ITS1 (forward): 5'-TCCGTAGGTGAACCTGCGG-3'
  - ITS4 (reverse): 5'-TCCTCCGCTTATTGATATGC-3'

The thermal cycling profile consisted of: 95 °C for 1 minute, followed by 36 cycles of 94 °C for 30 seconds, 53 °C for 20 seconds, 72 °C for 30 seconds. This was extended by a final elongation step at 72 °C for 5 minutes, and held at 15 °C until sample collection. Following amplification, the PCR products were electrophoresed on a 1% agarose gel, a DNA fragment of approximately 490 bp in size is suitable. The gene fragment was then sequenced by a sequencer and compared with the GenBank (NCBI) database using the BLAST SEARCH tool.

- The final identification of the endophytic fungi was confirmed by combining both traditional and molecular methods.

Phylogenetic tree construction method

All sequences were edited by removing gaps and missing data using the Chromas 2.5 software before being used for phylogenetic analysis in the MEGA7 software.

Diversity index analysis method

The diversity of endophytic fungi was analyzed using various indices such as the Simpson dominance index (D), the Simpson diversity index (1 - D), the Shannon diversity index (H'), the Margalef richness index (Dmg), the Menhinick richness index (Dmn), the Camargo dominance index (1/Dmn), and the Pielou evenness index (P).

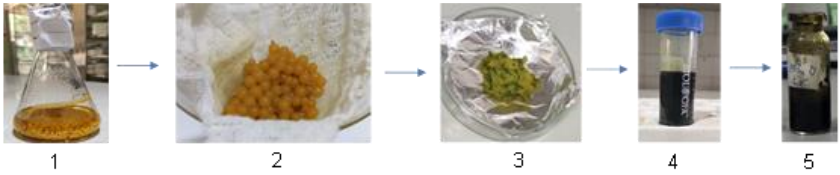
Method for determining biological characteristics

Physiological and biochemical characteristics were determined, including pH (5−8), temperature (22 °C −37 °C), and the ability to produce extracellular enzymes (cellulase acting on a 0.2% cellulose substrate and chitinase acting on a 0.2% chitin substrate). The diameter of the degradation halo reduced the turbidity of the medium, which became opaque white when stained with Lugol's solution.

2.2.4. Method for screening endophytic fungi strains producing podophyllotoxin and its derivatives

Method for fungal extract collection

Fungal strains were shaken/agitated in 100 mL of PDA medium at pH 7 at a rate of 150 rpm and 25±2 °C for 5 days. The fresh fungal biomass was then harvested by filtration, oven-dried at 45 °C −60 °C, and then soaked in 100 mL of methanol (an organic solvent) for 24 hours. The mixture was subjected to ultrasonic cell disruption for 1 hour, and this process was repeated 3 times to obtain the biomass extract.



1: fermentation; 2: filtration to collect wet fungal biomass; 3: dry fungal biomass; 4: total methanol extract; 5: fungal extract)

Method for determining podophyllotoxin and its derivatives

- *Thin Layer Chromatography (TLC)*: This is a rapid detection method for the presence of PTOX and its derivatives by analyzing 5 µL of the fungal extract on a 0.25 mm silica gel plate. The analysis was performed using a Dichloromethane:Methanol (9:1,v/v) solvent system. A podophyllotoxin standard (1 mg/mL in methanol) was used as a positive control. The silica gel plate was sprayed with 10% H_2SO_4 to visualize PTOX or examined under UV light at a 254 nm wavelength to detect the compounds in the extract.

- *High-performance liquid chromatography (HPLC*):

Analysis PTOX in the fungal extract was analyzed under the following conditions:

HPLC system: Agilent 1200 system equipped with a Diode array detector (DAD) (Agilent Technologies, Palo Alto, CA, USA).

DAD detector: Detection wavelength of 210 nm.

Chromatographic column: Eclipse XDB-C18 (250 mm×4.6 mm,5 µm).

Column temperature: 25 ºC.

Flow rate: 0.5 mL/minute

Sample concentration: 10 mg/ml

Standard concentration (Podophyllotoxin in methanol): 1 mg/ml

Injection volume:10 µl.

Mobile phase ACN-H_2O, solvent gradient elution program:

Time (minutes):	0	10	23	35	60
ACN (% volume):	10	20	50	90	90

The identification of PTOX and its derivatives was performed by comparing the retention time (Rt) and the UV spectrum with the PTOX standard.

2.2.5. Evaluation of cytotoxicity of endophytic fungal extracts

This assay was performed to determine the total cellular protein content based on the Optical density (OD) measured after staining the cellular protein components with sulforhodamine B (SRB).

2.2.6. Method for determining antibacterial activity of fungal extracts

The ability of the fungal extracts to inhibit bacterial growth was assessed using the agar well diffusion method. Accordingly, the fungal extracts and controls were loaded into sterile filter paper discs/wells and placed onto the same PDA medium plate that had been previously inoculated with bacteria. The diameter of the inhibition zone was measured in mm, and the experiment was repeated 3 times.

2.2.7. Method for genomic study of endophytic fungi strains producing podophyllotoxin

The genomes of the fungal strains were sequenced on the Pacbio Sequel system following the manufacturer's kit instructions.

Genome assembly and functional annotation: The sequenced genome was assembled and functionally annotated. Gene sets were used with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, employing the BLAST tool to retrieve KO (KEGG Orthology) annotation information from the KEGG database. The gene sequences were compared against the Carbohydrate-Active Enzymes database (CAZy) using BLAST to obtain

information regarding the species source, EC (Enzyme Commission) functional classification, gene and protein sequences, and the protein structure of CAZymes (Kanehisa et al., 2004), (Lombard et al., 2014).

Genes involved in the PTOX biosynthesis pathway in *Penicillium herquei* were identified using the UniProtKB database, which provided 10 target protein sequences, including 9 PTOX synthesis genes from the plant *Podophyllum hexandrum* and 1 viriditoxin synthesis gene from the fungus *VdtdD*. Subsequently, these sequences were used as "bait" to search for homologous genes within the genome of the fungus *Penicillium herquei* using the BLASTP tool on NCBI.

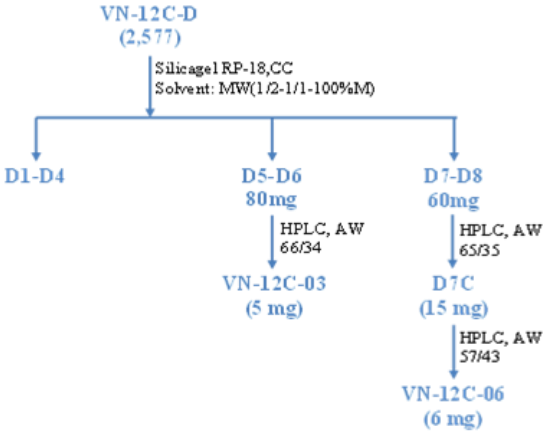
2.2.8. Method for determining appropriate fermentation conditions for fungal biosynthesis of podophyllotoxin and its derivatives

In this study, the factors investigated include: medium, inoculum age and inoculation ratio (or inoculum size), pH and temperature, aeration rate, carbon source, nitrogen source, mineral salts, and the influence of the precursor phenylalanine and a modified supplement of extract from *Podophyllum hexandrum* root (Medawar et al., 2003). From this, a kinetic study of the fermentation bioprocess for the biosynthesis of the podophyllotoxin compound/precursor and its derivatives was conducted to determine the precise termination time of the fermentation before the fungus undergoes degradation (or autolysis) which would reduce the content of the resulting podophyllotoxin compound/precursor.

2.2.9. Methods for isolation and structural elucidation of compounds from the fungal fermentation extract

- Isolation of compounds from the fungal extract

-The chemical structure of the compounds was determined by a combination of modern spectroscopic methods such as Mass Spectrometry (MS) (ESI-MS, HR-ESI-MS), one-dimensional (1D) Nuclear Magnetic Resonance (NMR) spectroscopy (^{1}H-, ^{13}C-NMR, DEPT), and (2D) two-dimensional (NMR) spectroscopy (HSQC, HMBC, ^{1}H-^{1}H-COSY)



2.2.10. Method for evaluating antioxidant activity

The antioxidant activity of the crude extract was determined by measuring its ability to neutralize DPPH free radicals according to Yuvaraj *et al.* (2013). The EC_{50} value (Effective Concentration at 50% - the concentration of the extract solution that neutralizes 50% of the DPPH free radicals) was determined using the TableCurve software. Samples with a lower EC_{50} value exhibit higher antioxidant activity.

CHƯƠNG 3. RESULTS AND DISCUSSION

3.1. Results of Bat giac lien sample collection

The research team randomly collected healthy plant samples from 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: 1074m) (2) Ngai Thau hamlet, Khun Ha commune, Tam Đuong district, Lai Chau province (22°13'2.55"N 103°35'0.58"E, độ cao: 1890m).

Figure 3.1. Stem and root of *Podophyllum* plant samples collected in Ha Giang and Lai Chau.

The plant samples were verified and classified by Trịnh Ngọc Bôn from the Vietnamese Academy of Forest Sciences and Nguyễn Thị Thanh Hương from the Plant Room, Institute of Biology. Accordingly, based on morphology, both samples were classified as the species *Dysosma diffiformis* (Hemsl. & E.H.Wilson) T.H.Wang (Synonym: *Podophyllum tonkinense* Gagnep.). The plant has a rhizomatous stem consisting of many nodes, with a rough structure, and it develops horizontally. The aerial stem usually bears one or two petiolate leaves. The leaf blade is wide, approximately 20 to 30 cm, with the petiole attached near the center of the blade. The leaf blade is divided into 6 to 8 shallow lobes; the lobes are broadly triangular or ovate, with pointed tips and small serrated margins. The collected samples grew under the canopy of primary forest.

3.2. Isolation of endophytic fungi

After surface sterilization procedures, endophytic fungi were obtained from the root (or rhizome), stem, and leaf samples of the BGL plant. From the two samples of *Dysosma diffiformis* collected in Hà Giang and Lai Châu, the research team isolated 53 endophytic fungal strains on PDA medium at **25** ^{0}C. Twenty-eight strains were isolated from the Hà Giang sample and 25

strains from the Lai Châu sample. The majority of the endophytic fungi were isolated from the root (or rhizome) samples with 82% (23 strains), followed by the leaf samples with nearly 11% (3 strains), and the stem samples with 7% (2 strains). For the Lai Châu sample, only four strains were found in the root (or rhizome) (14%), while the numbers isolated from the leaf and stem were eleven and ten, respectively (44% and 40%). The endophytic fungi isolated from the tuber were designated by the symbol (C), and those isolated from the root were designated by the symbol (R). For convenient identification and management, the research team agreed to convert the symbol (C) to (R) and the symbol (R) to (.1R). For example: HGN3C → HGN3R; HGN3R → HGN3.1R

3.3. Construction of the phylogenetic tree of microbial strains

From the 53 Endophytic Fungal Strains (EF) isolated, morphological characteristics were observed, including the color, shape, size, surface texture, and margin type of the colonies, the color of the aerial mycelia, the color of the underside (reverse side), and diffusible pigment into the medium. Combining this with sequential analysis, 53 EF strains were identified, belonging to 27 genera. The frequency of colonization for each isolated fungal species was calculated, showing that *Fusarium* is the most prevalent genus (11.11%), followed by *Trametes* (9.26%) and *Penicillium* (7.41%). Two phylogenetic trees detailing the taxonomic relationship among the endophytic fungal species isolated from the two collection sites were constructed using MEGA 7 software.

In Hà Giang, the endophytic fungal species formed two clusters: Cluster 1 contained genera belonging to the phylum *Ascomycota*, and cluster 2 contained genera belonging to the phyla *Mucoromycota* and *Basidiomycota*. Meanwhile, most of the genera belonging to the phylum *Ascomycota* were found in cluster 1 with *Mucor*, and the genera belonging to the phylum *Basidiomycota* were found in cluster 2. Only LCN12.1L was identified as *Penicillium herquei* 50SG10—a fungus belonging to the phylum *Ascomycota*—located in cluster 3. The difference in the phylogenetic tree structure between the two collection sites suggests that

varying habitats may influence the genetic evolution and diversity of the endophytic microbial species living in *D. diffiformis*.

3.4. Analysis of microbial diversity indices

The species diversity of endophytic fungal strains (VNNS) in the different plant tissues of *D. diffiformis* was analyzed in more detail through seven different diversity indices. Specifically, the Shannon diversity index showed the highest diversity in the root (H′ = 2,673), followed by the stem (H′ = 2,162) and the lowest in the leaf (H′ = 2,054). Similarly, taxonomic diversity in the root (Dmg = 4,551; Dmn = 3,079) and stem (Dmg = 4,024; Dmn = 3,175) was higher than in the leaf (Dmg = 3,41; Dmn = 2,405). However, the Simpson diversity index indicated that the endophytic population was highest in the leaf (1-D = 0,911), followed by the stem (1-D = 0,897) and the root (1-D = 0,867) (Bảng 3.4). Finally, the species richness index (S = 0,538) was highest when comparing the root with the leaf, which suggests that the number of endophytic species distributed between the leaf and root is higher than between the leaf and stem (0,286) or the stem and root (0,148) (Table 3.5). The Sorensen similarity index is 0,615, indicating a moderate level of taxonomic overlap between the two sites, Hà Giang and Lai Chau (Table 3.6). This is the initial report on the diversity of endophytic fungi isolated from *D. diffiformis*, opening up potential for screening endophytic fungal strains capable of producing important secondary metabolites.

3.5. Screening of fungal strains capable of biosynthesizing the active compound podophyllotoxin and its derivatives

3.5.1. Evaluation of the cytotoxicity of endophytic fungal extracts

The fungal extracts were evaluated for their cytotoxic potential, in order to identify potential strains for further in-depth research on the bioactive compounds within the fungal extract, as well as their mechanism of action. Accordingly, the lower the IC_{50} value (50% Inhibitory Concentration - the concentration that inhibits 50% of growth), the higher the toxicity of the fungal extract, meaning a lower concentration is needed to kill cancer cells.

The in vitro cytotoxic activity results of the fungal extracts were studied against three cancer cell lines (SK-LU1, HL-60, and HepG2)

compared to the *D. diffiformis* extract and positive controls such as podophyllotoxin and ellipticine. The results showed that twenty-eight extracts exhibited cytotoxic activity against SK-LU-1 cells with IC50 values ranging from 0.036 to 80.23 μg/ml (Table 3.7). These extracts were further investigated for anti-cancer activity against HL-60 (Human Acute Promyelocytic Leukemia) and HepG2 (Human Hepatocellular Carcinoma). We obtained six fungal extracts belonging to the genera *Penicillium* (two strains), *Trametes*, *Purpureocillium*, *Aspergillus*, and *Ganoderma* that showed strong cytotoxic activity against HL-60 and HepG2 with IC_{50} values ranging from 0.073 to 0.31 μg/ml and from 1.60 to 10.84 μg/ml, respectively (Table 3.8), while other extracts showed no cytotoxic activity (data not shown).

3.5.2. Development and screening of strain HGN12.1R for podophyllotoxin (PTOX) and its derivatives using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC)

Based on initial data, six fungal extracts—HGN13R, LCN8T, LCN3T, HGN12.1R, HGN12.2R, and HGN13.1R—which showed very strong cytotoxic activity against the three cancer cell lines, were further studied by rapid separation using Thin-Layer Chromatography (TLC). The results indicated that all six strains showed the presence of PTOX (Figure 3.5).

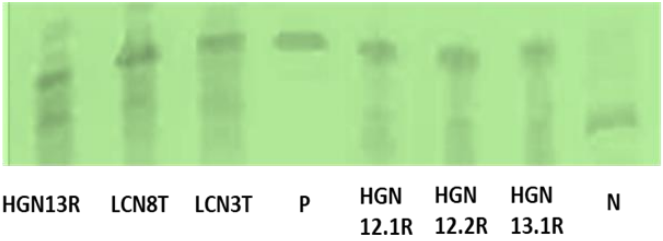
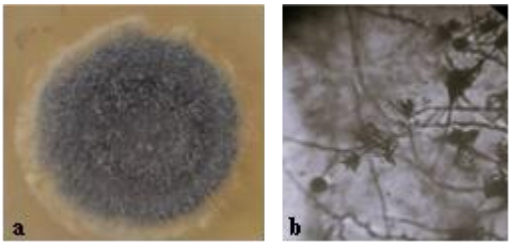


Figure 3.5. TLC analysis of the endophytic fungal extract. Podophyllotoxin was used as the standard, positive control (P); medium without fungus was used as the negative control (N)

HPLC analysis was then conducted to provide a more accurate tool for determining PTOX in the plant extract and fungal extracts. HPLC comparison of the two samples showed that the six fungal extracts all had a

retention time (Rt) identical to the PTOX standard. HGN12.1R is one of the most promising strains, exhibiting an initial capacity for compound production that rivals the plant source, as it showed the strongest cytotoxic activity on all three cancer cell lines. The combined analysis using TLC and HPLC UV spectra revealed that HGN12.1R exhibits a UV spectrum consistent with the PTOX standard, but also contains other peaks besides PTOX, suggesting the presence of complex secondary metabolites that hold great potential for high-value applications. Additionally, studies on the genus *Penicillium* have demonstrated that it is a source of PTOX and a large number of secondary metabolites with novel structures and bioactivity discovered from strains belonging to the genus *Penicillium*.

3.5.3. Biological characteristics of strain HGN12.1R



Colonies are moss green, circular, with a diameter of 6-8 mm. The surface of the aerial mycelia is fine like dust, the center is slightly raised (pulvinate), the margin is continuous and opaque white, and it does not produce diffusible pigment (a). The micro-morphological characteristics of the fungus (b) show septate hyphae, a penicillus-type conidiophore. The conidiophore surface is rough, with many fine, uniform spines (or granulations). The conidia are initially globose, then tear to form long, sparse chains of conidia. Preliminary identification of the fungal strain HGN12.1R through its color, colony morphology, and micro-morphological characteristics based on dichotomous keys placed it in the fungal genus *Penicillium*. Combining this with molecular biological methods led to the final result of identifying HGN12.1R as *Penicillium herquei* HGN12.1R.

The strain *Penicillium herquei* HGN12.1R grew optimally at 25 ^{0}C, pH 6-7. The *in vitro* antimicrobial activity was investigated using the agar

disk diffusion method (or agar diffusion method). The results showed that the fungal strain was effective against Gram-positive bacteria such as *Staphylococcus* sp., *Bacillus* 7TM11, *Bacillus* 2TM6, *Enterobacter* 14RM7-1, and appeared to have no or very little effect against the Gram-negative *Escherichia coli* DH5. This finding is consistent with previous studies where endophytic fungi from the genus *Dysosma* possess both anti-cancer and antimicrobial properties. Investigation of the extracellular enzyme activity of strain *P. herquei* HGN12.1R demonstrated the ability to degrade chitinase and cellulase. The average hydrolysis zone (or clear zone) for chitinase was 6.3±0.08 mm and for cellulase was 8.5±0.05 mm.

3.5.4. Results of the genomic characterization of endophytic Penicillium herquei HGN12.1R from Dysosma diffiformis and etabolic pathway prediction

The genome sequence of *Penicillium herquei* HGN12.1R was assembled, annotated, and analyzed using bioinformatics tools. The genome of strain HGN12.1R was fully sequenced and long reads were performed on the PacBio Sequel system. After integrating the de novo sequencing data, we determined the complete genome of *Penicillium herquei* HGN12.1R, with an approximate size of 34.96 Mbp across 9 contigs, 7 of which were larger than 1,000 bp. This genome contains all the necessary genes for protein synthesis and can be used for subsequent molecular biology and biotechnology applications research. The results of the fungal genome annotation determined the location and function of the genes and other genetic elements within the genome. This provides detailed information on the genes involved in the podophyllotoxin biosynthesis pathway. The Venn diagram (Figure 3.10) illustrates the overlap between the annotated gene sets when using OmicsBox, a comprehensive bioinformatics software that utilizes five databases: RefSeq Nonredundant (Nr), Uniprot, Gene Ontology, Pfam, and KEGG.

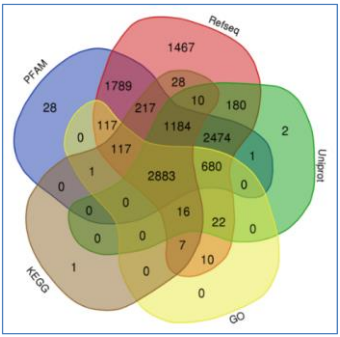


Figure 3.10. Functional gene snnotation Venn diagram

To identify the key genes related to the podophyllotoxin biosynthesis pathway in *Penicillium herquei* HGN12.1R, we screened and searched for candidate genes in the genome by performing a BLASTP search (with an E-value limit of 1×10−5) using 9 plant genes and 1 fungal gene (*VdtD* gene) as reference genes. Selecting the BLAST accessions with a threshold E-value of E<1×10−10 and verifying the results with the Pfam database, we found 33 annotated genes encoding 10 enzymes similar to those found in plants from the HGN12.1R genome. These include 5 SDH, 3 CYP71BE54, 5 CYP82D61, 6 CYP719A23, 5 CYP71CU1, 2 VdtD, 4 OMT3, 1 OMT1, 1 2-ODD và 1 PLR. Among these, VdtD is the only gene found in the fungus that has a function similar to the dirigent gene (DIR) in plants.

3.5.5. *Determining appropriate conditions for podophyllotoxin and its derivatives production from Penicillium herquei HGN12.1R using fermentation technology*

To determine the optimal conditions for the production of podophyllotoxin and its derivatives, we investigated the fermentation factors and obtained the following results: PDA medium with dextrose as the carbon source was selected, yielding the highest total soluble solids (SKK) at 14.09±0.06 g/l among the six media tested. Based on this result, we verified it with TLC analysis, which showed a similar result by detecting a diversity of bands when culturing *Penicillium herquei* HGN12.1R on PDA (Figure 3.12).

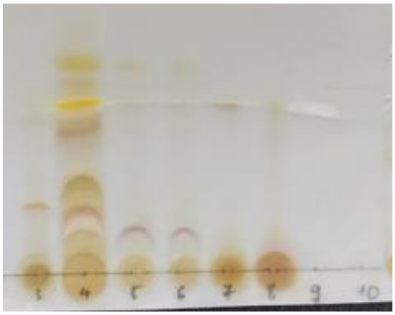


Figure 3.12. TLC analysis determining the presence of the compound/precursor in different media (lane 3: Czapek-Dox, lane 4: PDA, lane 5: Zhao, lane 6: YMA, lane 7: PGA, lane 8: Sabouraud broth, lane 9: PTOX standard compound, lane 10: negative control (DC-)).

Penicillium herquei HGN12.1R is suitable at a pH range of 5-7 and at 25 °C on PDA medium. Studying the inoculum age and inoculation ratio is crucial as these factors significantly influence the strain growth rate, fungal density, and pellet size. The 48-60 hour time frame is when the fungus grows quickly and vigorously with high fungal density, making it the appropriate time to transfer the fungus to the fermentation medium to create conditions for continued development and better PTOX biosynthesis. Combining this with an inoculation ratio of approximately 5% leads to uniformly sized pellets, preventing clumping, and the circulating flow increases the ability to absorb nutrients and exchange oxygen.

Investigating the shaking speed and culture volume are key conditions that provide better dissolved oxygen to the medium and ensure the fungal mycelia are thoroughly mixed, resulting in uniform contact with the medium from top to bottom. The study reveals that the strain *Penicillium herquei* HGN12.1R is suitable for a shaking speed of 150 rpm and a medium volume equal to 20% of the total container volume.

Cultivation conditions for *Penicillium herquei* HGN12.1R were optimized for efficient mycelial biomass and PTOX biosynthesis. Dextrose was determined to be the most suitable carbon source, while the fungus exhibited a preference for organic nitrogen sources, allowing corn steep liquor (CNM) to be replaced by beef extract, peptone, or casein hydrolysate. For mineral nutrition, the fungus *Penicillium herquei* HGN12.1R was suitable for the mineral source $MgSO_4 \cdot 7H_2O$, yielding higher biomass content compared to K_2HPO_4 and KCl.

Recognizing the inherently low yield of secondary metabolites in fungal fermentation, the process was further enhanced by adding stimulators. Both plant extract and phenylalanine successfully promoted strain growth and increased PTOX yield. Ultimately, phenylalanine was selected as the preferred stimulator for biosynthesis enhancement due to the conservation status of the source plant for the alternative extract.

Based on the sufficient establishment of all optimal fermentation factors, the fermentation kinetic of the *Penicillium herquei* HGN12.1R strain was performed to determine the appropriate harvest time. The test results showed that at approximately 168 hours (h), the PTOX content and its derivatives, along with the biomass yield, were high and stable. From this point, the research group harvested the culture, performed extraction, purification, and structure elucidation of the compounds from *Penicillium herquei* HGN12.1R. After the liquid-liquid extraction process using the organic solvents n-hexane, ethyl acetate, and methylene chloride sequentially, we obtained various fractions. The fractions that showed similar bands on the Thin-Layer Chromatography (TLC) plate were pooled and subjected to High-Performance Liquid Chromatography (HPLC) (Agilent 1260 Infinity II), yielding two compounds: VN12C - 03 (5 mg) and VN12C - 06 (6 mg).

The aqueous phase was subjected to Diaion HP-20 column chromatography, yielding three fractions labeled W1, W2, and W3. However, using a combination of chromatographic methods such as TLC, Column Chromatography (CC), and HPLC for these aqueous fractions and other fractions did not result in the isolation of any pure compounds.

3.5.6. Determination of the molecular structure of compounds VN-12C-03 and VN-12C-06

Compound VN-12C-03 was obtained as a white powder. NMR ^{1}H analysis, together with ^{13}C NMR and 2D HSQC spectra, showed that VN-12C-03 possesses a lignan skeleton — a relatively common phenolic type found in plants. Comparing the ^{1}H and ^{13}C NMR data of VN-12C-03 with that of the known lignan α-Peltatin yielded perfectly matching results, suggesting structural identity between the two compounds. Based on comparison with reference materials, compound VN-12C-03 was identified

as α-Peltatin, with a molecular formula of $C_{21}H_{20}O_8$, a molecular weight of 400 g/mol, and is considered a derivative of podophyllotoxin.

Compound VN-12C-06 was obtained as a white powder. Signals from the ^{1}H NMR spectrum, the ^{13}C NMR spectrum, and the HSQC spectrum suggested that compound VN-12C-06 possesses a lignan skeleton, similar to compound VN-12C-03. Based on the results of the spectral analysis and comparison with reference materials, compound VN-12C-06 was identified as β-Peltatin, with a molecular formula of $C_{22}H_{22}O_8$ and a molecular weight of 414 g/mol. β-Peltatin is also considered a derivative of podophyllotoxin.

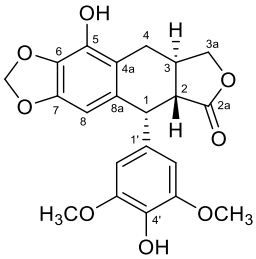


Figure 3.29. Molecular structure of compound VN-12C-03

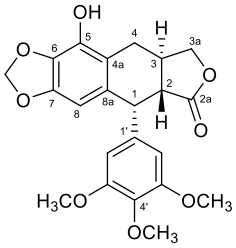


Figure 3.30. Chemical structure of compound VN-12C-06

3.5.7. Evaluation of the iiological activity of the two compounds VN12C - 03 and VN-12C – 06

Results of the assessment of antioxidant activity using the DPPH free radical scavenging method

The ability of the test samples to neutralize DPPH free radicals was demonstrated by the reduction in the color of DPPH, which was determined by measuring the absorbance at 517 nm using an ELISA reader. In general, for all compounds (VN12C.03, VN12C.06) and the standard (Resveratrol), the percentage of DPPH free radical inhibition increased as the concentration increased. This is consistent with the mechanism of action of antioxidants, where higher concentrations provide more molecules to neutralize the free radicals.

Results of the Cytotoxicity Assay

Results of cytotoxicity against the SK-LU-1 cell line: Human lung carcinoma. The mean percentage of cell inhibition (CI) and the corresponding standard deviation (or error) at various concentrations (20;4;0.8;0.16;0.032 μg/ml) for each tested sample are reported. The results showed that the tested samples exhibited cytotoxic activity against the SK-LU-1 cell line with IC_{50} values ranging from 0.04−18.61 μg/ml. The positive control, Ellipticine, performed consistently in the experiment. In general, the percentage of inhibition tended to decrease as the concentration decreased.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSION

1. 53 endophytic fungal strains were isolated from two samples of the plant Dysosma difformis collected from Ha Giang and Lai Chau provinces. Among these, 06 strains were selected for exhibiting very strong cytotoxic activity against three cancer cell lines: SK-LU-1 (Human lung carcinoma), HL-60 (Human acute leukemia), and HepG2 (Human hepatocellular carcinoma). Combining analytical methods such as TLC and HPLC to determine their ability to synthesize PTOX compounds/derivatives, the HGN12.1R strain was selected as the potential candidate for subsequent studies.
2. The genome of the fungal strain *Penicillium herquei* HGN12.1R was sequenced and assembled using PacBio technology. The *Penicillium herquei* HGN12.1R genome size is approximately 35.0 Mb, corresponding to a completeness of 98.3%. The genome was annotated, and gene clusters related to the biosynthetic pathway of podophyllotoxin and its derivatives were predicted.
3. The optimal conditions for the production of podophyllotoxin and its derivatives by the *Penicillium herquei* HGN12.1R strain were identified using laboratory-scale fermentation technology with the following parameters: 5% inoculum size; pH 7.0; temperature of 25 °C; agitation speed of 120 rpm; aeration rate of 1 vvm; and an optimal fermentation time of 144 hours.
4. The structure of 02 compounds, α-peltatin and β-peltatin, which are derivatives of podophyllotoxin, were isolated, purified, and structurally

elucidated. Both compounds exhibited antioxidant activity and cytotoxic activity with EC_{50} values ranging from 6,44±0,41 ÷ 18,75±1,11 µg/mL, and cytotoxic activity with IC_{50} values ranging from 5,99±0,41 ÷ 18,61±0,41 µg/mL.

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RECOMMENDATIONS

- Continue research to optimize fermentation conditions and conduct further studies to increase the biosynthetic yield of PTOX and its derivatives in the *Penicillium herquei* HGN12.1R strain.
- Evaluate the function of predicted genes involved in the biosynthetic pathway of PTOX and its derivatives in the studied fungal strain.
- Conduct in vivo experimental studies on the activity of VN12C.03 and VN12C.06.
- Assess the cytotoxic activity of VN12C.06 against various cancer cell lines to determine its selectivity and activity spectrum.

NOVELTY CONTRIBUTIONS OF THE DISSERTATION

This thesis presents the first systematic study on the isolation, identification, and evaluation of the ability of endophytic fungal strains isolated from the medicinal plant *Dysosma differmis* (Bát Giác Liên) to produce the active substance podophyllotoxin and its derivatives. The preliminary results of this study indicate that generating an alternative source of raw materials not only alleviates pressure on natural resources but also demonstrates high potential for practical application. This establishes a foundation for developing efficient podophyllotoxin production processes to serve the pharmaceutical industry.

The main specific points achieved are:

- Six endophytic fungal strains were isolated from *Dysosma differmis* samples in Vietnam, demonstrating strong cytotoxic activity against cancer cells. Furthermore, the diversity of endophytic fungi in the local *Dysosma differmis* plant was assessed.
- The endophytic fungal strain *P. herquei* HGN12.1R was selected for its potential to synthesize PTOX. Its genome was sequenced using PacBio technology, annotated, and the biosynthetic pathway of podophyllotoxin and its derivatives was predicted.
- The structure of 02 compounds, α-peltatin and β-peltatin, which are derivatives of podophyllotoxin, were isolated, purified, and structurally elucidated from the endophytic fungal strain *P. herquei* HGN12.1R, and their biological activities were proven.

LIST OF PUBLICATIONS RELATED TO THE THESIS

- ***Scientific publications:***

1. **Hoa Thi Tran**, Giang Thu Nguyen, Hong Ha Thi Nguyen, Huyen Thi Tran, Quang Hong Tran, Quang Ho Tran, Ngoc Thi Ninh, Phat Tien Do, Ha Hoang Chu & Ngoc Bich Pham (2022). "*Isolation and Cytotoxic Potency of Endophytic Fungi Associated with Dysosma difformis, a Study for the Novel Resources of Podophyllotoxin*". Mycobiology, 50(5):389-398. DOI: 10.1080/12298093.2022.2126166.

2. **Tran Thi Hoa**, Nguyen Thu Giang, Nguyen Thi Hong Ha, Tran Thi Huyen, Do Tien Phat, Chu Hoang Ha, Pham Bich Ngoc, Tran Ho Quang (2023). "*Diversity of endophytic fungi from medicinal plants Dysosma difformis (Hemsl & E.H. Wilson) T.H. Wang collected in Ha Giang and Lai Chau"*. Vietnam Journal of Biotechnology, 21(2): 365-373. DOI:10.15625/1811-4989/18344.

3. **Tran Thi Hoa**, Nguyen Thi Hong Ha, Nguyen Thu Giang, Tran Ho Quang, Do Tien Phat, Chu Hoang Ha, Pham Bich Ngoc (2023). "*Survey of the antimicrobial activity of the endophytic fungus HGN12.1R extract isolated from the bat giac lien medicinal plant (Dysosma difformis (Hemsl & E.H. Wilson) T.H. Wang)*". Scientific Report - The National Biotechnology Conference 2023. Publishing House for Science and Technology: 540-544.

4. Duong Huy Nguyen , Quang Ho Tran, Lam Tung Le, Ha Hong Thi Nguyen, **Hoa Thi Tran**, Thuy Phuong Do, Anh Ngoc Ho, Quang Hong Tran, Hien Thi Nguyen Thu, Van Ngoc Bui, Hoang Ha Chu, Ngoc Bich Pham (2024). "*Genomic characterization and identification of candidate genes for putative podophyllotoxin biosynthesis pathway in Penicillium herquei HGN12.1C*", Microb Biotechnol, 17(9):e70007. DOI: 10.1111/1751-7915.70007.

- ***Utility Solutions:***

5. Utility Solution Patent for "The pure strain of fungus *Penicillium herquei* HGN12.1C, biologically capable of biosynthesizing the active compound Podophyllotoxin". Authors: **Tran Thi Hoa**, Nguyen Thi Hong Ha, Tran Thi Huyen, Tran Ho Quang, Do Tien Phat, Pham Bich Ngoc. Application No.: 2-2021-00393. Patent No.: 3352 under Decision No. 66238/QD-SHTT dated September 05, 2023, of the National Office of Intellectual Property.