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Isolation and identification of endophytic fungi from *Dendrobium huoshanense* with their antibacterial and anti-inflammatory activities

Jing-Yi Yue[1] and Yang Yu[2]*
[1]Wuhu Institute of Technology, Wuhu, PR China
[2]School of Pharmacy, Anhui University of Chinese Medicine, Hefei, China

Abstract: To examine the symbiotic relationship between *Dendrobium huoshanense* and its endophytic fungi, a total of 168 endophytic fungi were isolated and classified into 18 genera and 36 species via using pure culture and techniques of molecular biology. Among them, *Stagonosporopsis oculihominis*, *Alternaria eichhorniae*, *Phyllosticta aristolochiicola*, *Aspergillus flavus* and *Fusarium lactis* were the dominant genera. Further, the secondary metabolites produced by the dominant genera were screened for antibacterial and anti-inflammatory activities. The secondary metabolites of *Stagonosporopsis oculihominis* showed good inhibitory activity against *Staphylococcus aureus* and the release of nitric oxide (NO). Three benzene ring derivatives were also identified using chromatographic and spectroscopic techniques, including (Z)-1- hydroxy-4-(2-nitroethenyl)-benzene (1), p-hydroxybenzaldehyde (2) and p-hydroxyphenylacetic acid (3). These findings indicated that the endophytic fungi of *Dendrobium huoshanense* are diverse and their dominant genera exert similar anti-inflammatory and antibacterial effects as those of the host plant, which provided a scientific basis for the subsequent investigation of the medicinal value of *Dendrobium huoshanense*.

Keywords: *Dendrobium huoshanense*, endophytic fungi, diversity, secondary metabolites, antibacterial and anti-inflammatory activities.

INTRODUCTION

Endophytic fungi are fungi that infest and colonize healthy plant tissues at some stage of their life history without causing an obvious disease in the host plant; these include the saprophytic fungi, epiphytic in some stages of their life history, latent pathogens and mycorrhizal fungi that are harmless to the host plant on a temporary basis (Debary, 1866; Fisher and Petrini, 1992). Endophytic fungi and their hosts have a mutually beneficial symbiotic relationship, which forms the basis of the endosymbiosis theory as follows: endophytic fungi that have co-evolved with their hosts may receive directly transmitted relevant genes and have the same secondary metabolite synthesis pathways as their hosts (Petrini, 1991; Ahlholm *et al*., 2002; Hite *et al*., 1996), therefore, generating similar or the same secondary metabolites (Stierle, 1993).

Dendrobium huoshanense or Huoshan Mihu, is the most valuable representative species of *Dendrobium* and is the most famous regional drug of the Anhui Province (Zhao *et al*., 2021). Modern pharmacological research shows that *Dendrobium huoshanense* contains polysaccharides, alkaloids and flavonoids, which are effective in improving human immunity, exerting anti-oxidative, anti-tumor, hypoglycemia, anti-inflammatory and anti-bacterial effects and repairing liver damage (Mo *et al*., 2019). To date, its biological activity has mainly been reported for its polysaccharides or total extract, whereas studies on small molecule active substances have rarely been published (Su *et al*., 2020). Due to extremely harsh growing conditions and scarcity of wild resources, artificially cultivated *Dendrobium huoshanense* is expensive. Therefore, collecting a large number of samples for studies on chemically active ingredients is costly.

Given the endosymbiosis theory, seven batches of *Dendrobium huoshanense* in different cultivation environments were collected and their endophytic fungi were isolated and purified by tissue isolation. Further, the identification of the isolated endophytic fungi was performed using the techniques of molecular biology and biodiversity and dominant genera were determined using molecular evolutionary genetic analysis software. The dominant genera were identified by screening for the anti-inflammatory and antibacterial activities of the secondary metabolites of the dominant genera for initial isolation and identification of their secondary metabolic molecules. Finally, the biofunctional active substances obtained were found to be similar to those of the host plant. The findings of this study are expected to provide a new way to systematically reveal the symbiotic relationship between *Dendrobium huoshanense* and its endophytic fungi in the future.

MATERIALS AND METHODS

Instruments, reagents and cultivation

The instruments used in this study include JJ-CJ-2FD clean bench (Wujiang Purification Equipment General Factory, Jiangsu, China), MIKRO 22R high-speed frozen centrifuge (Hettick, Germany), SHP-250 biochemical incubator (Shanghai Jinghong Experimental Equipment, Shanghai, China), LDZX-30KBS vertical pressure-steam

**Corresponding author:* e-mail: ywhxyu@126.com

sterilizer (Shanghai Shenan Medical Equipment, Shanghai, China), 752 UV spectrophotometer (Shanghai Jinghua Instrument, Shanghai, China), T-Gradient Thermoblock PCR instrument (Biometra, Germany), DHG-9076A electric thermostatic blast oven (Shanghai Jinghong Experimental Equipment, Shanghai, China), DYY-8C electrophoresis apparatus (Beijing Liuyi Instrument Factory, Beijing, China) and Bruker AVANCE III 600MHz and 500MHz superconducting nuclear magnetic resonance system.

The reagents used in this study include plant/fungal genomic DNA small extraction kit (Shanghai Laifeng Biotechnology, Shanghai, China), potato dextrose agar medium (200g potato, 20g dextrose and 18g agar powder in 1000 mL distilled water), universal primers: ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) (Sangon Biotech, Shanghai, China), *Staphylococcus aureus subsp. Aureus* ATCC29213, *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853 and *Salmonella enterica subsp. enterica* ATCC14028 (China General Microbiological Culture Collection Center), ceftazidime (Shanghai Yuanye Biotech, Shanghai, China), penicillin G sodium (Biosharp), DMSO (Sigma), LB broth and MH broth (Guangdong Huankai Microbiology Technology, Guangdong, China), agar powder (Scientific Research Special). 1,1-diphenyl-2-trinitrophenylhydrazine (DPPH), mouse mononuclear macrophages RAW264.7 (Shanghai Cell Bank, Chinese Academy of Sciences), fetal bovine serum, Dulbecco’s modified eagle medium (DMEM) (BI) and control drug L-NMMA, LPS and Griess Reagent (Sigma).

Seven batches of whole three-year-old *Dendrobium huoshanense* plants cultivated in seven different environments were collected in December 2015, including (1) understorey cultivation CL at the base of Changchong Chinese Medicine Development Co. Ltd. (BCCMD) (North 31″03′23.91, East 115″59′33.02); (2) sawdust cultivation CM at BCCMD (North 31″04′28.16, East 115″58′45.68); (3) gravel cultivation CS at BCCDM (North 31″15′34.56, East 115″57′56.03); (4) understorey cultivation SL at Shengnong Shihu Development Co. Ltd.(SNSHD) (North 31″22′11.57, East 116″13′41.45); (5) sawdust cultivation SM at SNSHD (North 31″23′78.26, East 116″13′56.03); (6) gravel cultivation SS at SNSHD (North 31″23′20.64, East 116″13′56.03) and (7) sawdust cultivation PL at Pengze Biotech Co. Ltd. (North 31″25′18.46, East 116″16′15.98) These plants were identified by Professor Fang Chengwu of the Bozhou Vocational Technology College as *Dendrobium huoshanense*, belonging to the genus of *Dendrobium* and *Orchidaceae* family.

Isolation and purification of endophytic fungi

Disease-free wild *Dendrobium huoshanense* plants were rinsed well under running water. Routine surface disinfection was performed after the absorption of water from the surface of the fibrous roots and decaying leaves (Wang *et al*., 2007; Ratnaweera *et al*., 2018). Specifically, the plants were disinfected with 75% ethanol for 30s, sodium hypochlorite (1% available chlorine) for 1-3min, 75% ethanol for 1-2min and finally rinsed 2-3times using sterile water. After aspiration of the surface water, 200μL sterile water was added to the PDA medium in parallel triplicates as the blank control. Samples were placed upside down in an incubator and maintained at a constant temperature of 28°C for 3-5days. Control plates were free of colonies and complete surface sterilization was ensured. The roots and stems of the surface-sterilized plants were cut into 0.2cm long pieces using a sterilized blade. In addition, the leaves were cut into 0.1x0.1cm^2 pieces using sterilized scissors and inoculated in the PDA medium in parallel triplicates (3-5pieces/plate). The plates were then placed upside down in an incubator at a constant temperature of 28℃ for 5-15d and protected from light. Further, the mycelium (colonies) growing from the incision of the material in the Petri dishes was picked from the edge of each plate using a sterilized toothpick and placed in the new PDA medium. Finally, the culture was continued for two to three generations at conditions as described above to ensure that the resulting bacteria were pure.

Identification of molecules produced by the endophytic fungi (Chen et al., 2012)

DNA extraction from endophytic fungi: The purified strains were inoculated on PDA plates and incubated at 28℃ for 5-7days and protected from light. After the plates were covered with mycelium, with a scraper, the mycelium was scrapped and ground in a sterilized mortar. The DNA was extracted following the kit protocol for fungal genomic DNA extraction and stored at -20℃ until subsequent use.

PCR amplification of rDNA-ITS: The following primers used to amplify the fungal rDNA-ITS sequences: downstream ITS4: 5′-TCCTCCGCTTATTGATATGC-3′ and upstream ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′. The 50μL PCR reaction system consisted of the following: 25μL of endonuclease (Premix Tap), 2μL of ITS4 (20μmol/L), 2μL of ITS1 (20μmol/L), 19μL of double-distilled water and 2μL of DNA template.

The PCR amplification procedure involved pre-denaturation, 95°C for 5 min; denaturation, 95°C for 1 min; renaturation, 49°C for 1 min; extension, 72°C for 2 min (30 cycles) and final extension, 72°C for 10 min. The amplified PCR products were electrophoresed on 1% agarose gels in 1×tris (hydroxymethyl) aminomethane-acetic acid (TAE) electrophoresis buffer at 100 V for 40min and stained with ethidium bromide (EB) for 5min. Finally, the amplification products were verified and photographed on a gel imaging system and stored at -20°C until subsequent experiments.

Phylogenetic analysis of rDNA genes: The PCR amplified products were sequenced by Shanghai Sangon Biotech. The results of sequencing were compared against published sequences in the GenBank database. Subsequently, a phylogenetic tree was generated using MEGA ver.5.0, a molecular evolutionary genetic analysis software. The phylogenetic matrix was determined using the Kimura 2-Parameter model. Subsequently, the phylogenetic tree was generated by the neighbor-joining clustering method. Bootstrap value analysis was performed 1000 times to assess the stability of the topology of the phylogenetic tree. Finally, the strains were identified in combination with sequence comparisons.

Analysis of endophytic fungal diversity and determination of the dominant genera

Several factors, including colonization rate (CR), isolation rate (IR), isolation frequency (IF), Shannon index, diversity index, Sorenson similarity coefficient and Pielou homogeneity index (Qi *et al*., 2015; Zang *et al*., 2014), were determined and the distributional characteristics and diversity of the endophytic fungi of *Dendrobium huoshanense* were analyzed as follows:

CR(%)=number of contaminated tissues/total number of tissues in the sample×100%, represented the percentage of endophytic fungi-contaminated tissues to the total number of tissues in the sample; IR(%)=number of isolated fungi/number of tissues in the sample, indicating the ratio of the number of strains obtained from the tissues in the sample to the total number of tissues in the sample and IF(%)=number of isolates of each genus of endophytic fungi in the sample area/total number of endophytic fungi isolated in the sample area×100%, representing the percentage of the number of endophytic fungi isolated from the sample to the total number of isolated strains and was the direct indicator of the dominant genera/genus (genus with RF>10% 10% was defined as the dominant genus in a sample area, 5%≤RF≤10% as the common genus and RF<5% as a rare genus). Shannon index (H) was calculated as follows:

$$H = -\sum_{i=1}^{k} Pi \times \ln Pi$$

where *k* is the total number of endophytic fungal species in *Dendrobium huoshanense* tissues and *Pi* is the number of strains of a certain endophytic fungus as a percentage of all strains of endophytic fungi.

The diversity index (D) was calculated according to the Margaef index as follows: $D=(S-1)/lnN$, where *S* is the number of endophytic fungal species and *N* is the total number of endophytic fungal strains isolated. The Sorenson similarity coefficient (Cs) was calculated as follows: $Cs=2j/(a+b)$, where *j* is the number of endophytic fungal species common in each tissue of *Dendrobium huoshanense* or in different cultivation environments, *a* is the number of endophytic fungal species in one type of tissue or cultivation environment and *b* is the number of endophytic fungal species in another type of tissue or cultivation environment. Pielou evenness index (J) was calculated as follows: $J=H/InS$, where *S* is the number of endophytic fungal species and *H* is the diversity index.

The SPSS 22.0 statistical software was used to perform systematic cluster analysis. The similarity was measured according to the between-groups linkage method based on the square of the Euclidean distance. The results of the systematic clustering analysis were illustrated in a dendrogram.

Preparation of secondary metabolites produced by the dominant genera

The dominant genus assessed by biometrics was placed in an ultra-clean bench, inoculated in PDA medium and incubated in an incubator at a constant temperature of 28°C for 5-7 days. After punching with a hole puncher, the fungi (cake) were inoculated into conical flasks containing sterilized solid (rice) medium and incubated in an incubator at a constant temperature of 28°C for 28 days. The cake was then crushed and dried and extracted thrice using ethyl acetate. The extracts were concentrated and dried to obtain ethyl acetate extracts for the eight tested fungi.

Anti-bacterial assay for secondary metabolites of the dominant genus

(Wang *et al*., 2021; Waqar-ul-Konain *et al.*, 2022; Ali *et al*., 2022): The samples were tested for their potential antibacterial activity against 4 bacterial strains (*Escherichia coli* ATCC25922, *Staphylococcus aureus* subsp. aureus ATCC29213, *Salmonella enterica* subsp. enterica ATCC14028 and *Pseudomonas aeruginosa* ATCC27853 (China General Microbiological Culture Collection Center)) at a single concentration of 100µM. The inocula of the bacterial strains prepared from 24-h-old cultures and suspensions were adjusted to 0.5M cFarland standard turbidity. The 96-well plates were prepared by dispensing 100µl of Muller Hinton broth/Luria-Bertani into each well. A volume of 100µl aliquot from the stock solutions of the samples initially prepared was added to the first wells. Then, 100µl of inoculum was added to achieve a final inoculum concentration of 5×105 CFU/ml. The final volume in each well is 200µl. After an incubation at 37°C for 24hours, growth was monitored by a microplate reader at 625nm. The calculation was as follows:

Inhibition rate= (OD_{625nm} of drug-free control wells-OD_{625nm} of drug solution wells)/OD_{625nm} of drug-free control wells×100% (Ren *et al*., 2021)

Anti-inflammatory assay for secondary metabolites of dominant genera

(Laksmitawati *et al*., 2017): RAW264.7 cells were inoculated into 96-well plates and induced with 1µg/mL

LPS. Subsequently, the compound to be tested was added to a final concentration of 50μg/ml. L-NMMA-positive drug and drug-free groups were set as controls. NO production was detected after overnight incubation of the cells and absorbances at 570 nm were measured. Cell viability assays were performed after the addition of MTS to the remaining medium to exclude the toxicity due to the compounds on the cells.

NO production inhibition (%)=(OD_{570nm} for drug-free group-OD_{570nm} for sample group)/OD_{570nm} for drug-free group×100% (Yu *et al.*, 2022)
50% concentration of inhibition (IC50) was calculated according to the method of Reed & Muench.

Preliminary studies on secondary metabolites of Stagonosporopsis oculihominis

The solid medium with completely fermented *Stagonosporopsis oculihominis* strain was removed and crushed. The crushed samples were extracted 5-6 times after natural drying by soaking in ethyl acetate, shaking and sonication. Each extraction concentrate was pooled and concentrated under reduced pressure until it was dry to obtain 90g of crude ethyl acetate extract. A forward silica gel column (200-300 mesh) was used to elute the delineated segments with dichloromethane-methanol (1:0-0:1, v/v) as the eluent. Subsequently, the monomeric compounds 1-3 were obtained by repeated liquid-phase separation using reverse chromatography and gel and HPLC analysis. Finally, the nuclear magnetism and mass spectrometry data were compared with the reported values and these compounds were identified (Wang *et al.*, 2021; Yu *et al.*, 2017).

RESULTS

By tissue isolation, 168 endophytic fungi were isolated from the seven batches of *Dendrobium huoshanense* cultivated in different environments and classified into 18 genera and 36 species using methods of molecular biology. For the isolated endophytic fungi, a phylogenetic tree was constructed according to the calculations of isolation frequencies of each genus/species (table 1) by neighbor-joining clustering (fig. 1). *Stagonosporopsis oculihominis*, *Alternaria eichhorniae*. *Phyllosticta aristolochiicola*, *Aspergillus flavus* and *Fusarium lactis* were the dominant species in *Dendrobium huoshanense*, while Colletotrichum was the common genus and the remaining were rare genera. Among them, *Stagonosporopsis oculihominis* and *Alternaria eichhorniae* were isolated from four environments; *Phyllosticta aristolochiicola* and *Aspergillus flavus* were isolated from three environments and *Fusarium lactis* was isolated in two environments. Additionally, the genera *Coniothyrium*, *Pleospora*, *Botrytis*, *Bionectria*, *Ulocladium*, *Curvularia*, *Wojnowiciella* and *Acrocalymma* were isolated in only one environment, while *Stagonosporopsis* and *Fusarium* were isolated from five and six cultivation environments, respectively. Therefore, the endophytic fungi isolated from *Dendrobium huoshanense* in different cultivation environments were closely related to the geographical habitats of their host and displayed some geographical specificity.

Screening for antibacterial and anti-inflammatory activities of secondary metabolites produced by the dominant genera

Based on the endosymbiosis theory, the antibacterial and anti-inflammatory activities of the host plant were used to screen the antibacterial, antioxidant and anti-inflammatory activities of the secondary metabolites produced by the five dominant genera. As shown in tables 2-3, the secondary metabolites of *Stagonosporopsis oculihominis* exhibited good inhibition of *Staphylococcus aureus* at 128μg/ml relative to the other four dominant genera. Additionally, it also showed good inhibition of NO production at 50μg/ml.

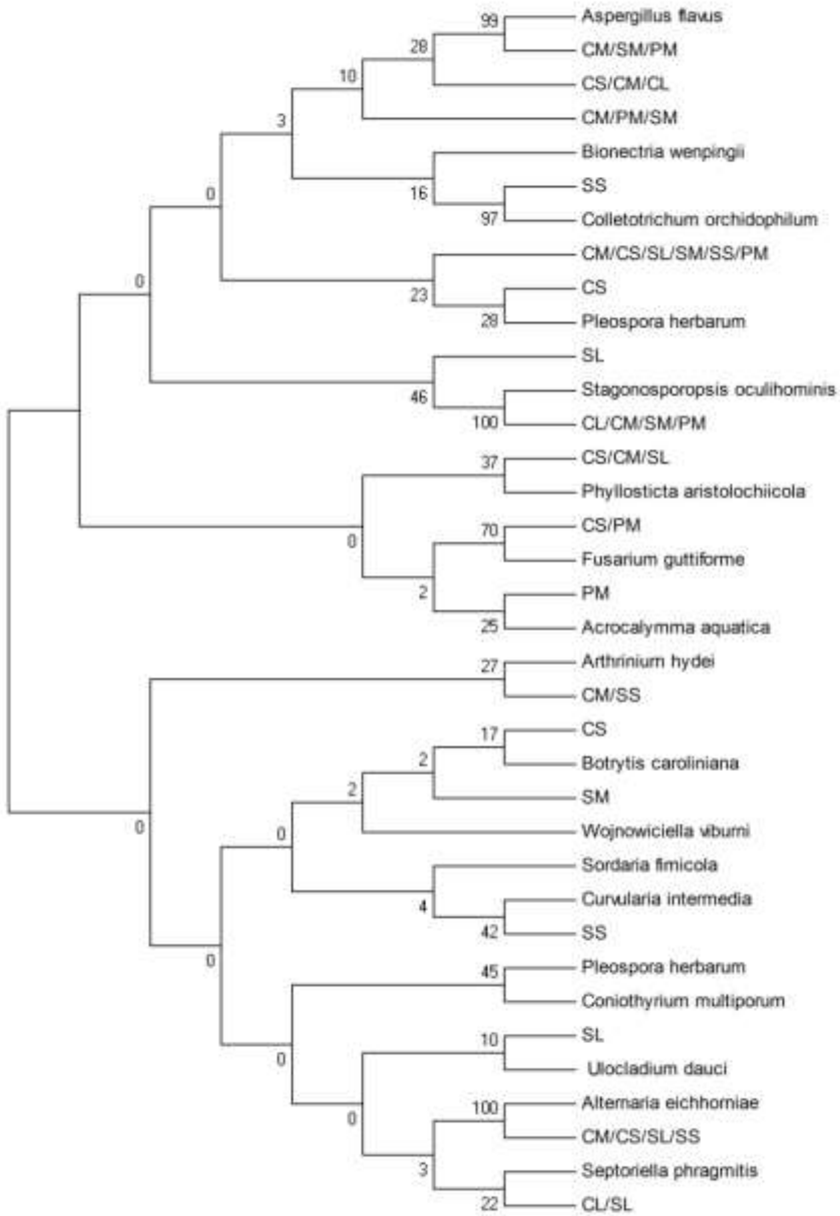
Compound 1: $C_8H_7NO_2$, white powder; ^{1}H-NMR(600MHz, $CDCl_3$) δ: 6.96 (2H, d, *J*=8.0 Hz, H-2, H-6), 7.72 (2H, d, *J*=8.0 Hz, H-3, H-5), 7.88 (1H, d, *J*=13.5 Hz, H-8), 8.03 (1H, d, *J*=13.5 Hz, H-7), 9.07 (1H, brs, OH-1); ^{13}C NMR (150MHz, $CDCl_3$) δ: 162.2 (C-1), 117.2 (C-2, C -6), 132.8 (C-3,C-5), 122.9 (C-4), 140.1 (C-7), 135.9 (C-8). These data followed the previously reported values (Liao *et al*., 2017). Therefore, compound 1 was determined to be (Z)-1- hydroxy-4-(2-nitroethenyl)-benzene.

Compound 2: $C_7H_6O_2$, colorless powder; ^{1}H-NMR (500 MHz, $(CD_3)_2CO$) δ: 6.26(1 H, s, OH), 6.75 (2H, d, *J*=8. 7 Hz, H-3, H-5), 7.79 (2H, d, *J*=8.7 Hz, H-2, H-6), 9.76 (1H, s, CHO);^{13}C NMR (125MHz, $(CD_3)_2CO$) δ: 116.6(C-1), 132.8(C-2, C-6), 130.4(C-3, C-5), 163.8(C-4), 191.0(C-7). These data were in general agreement with published literature (Cai *et al*., 2020). Therefore, compound 2 was determined to be p-hydroxybenzaldehyde.

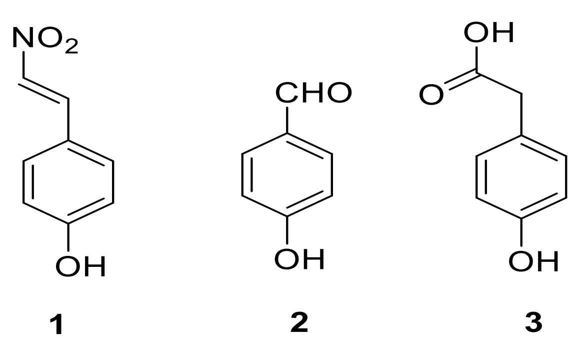
Compound 3: $C_8H_8O_3$, white needle-like crystals; ^{1}H-NMR (600MHz, CD_3OD) δ: 3.36 (2H, s, H-7), 6.68 (2H, d, *J*=8.6Hz, H-3, H-5), 7.12 (2H, d, *J*=8.3 Hz, H-2, H-6); ^{13}C-NMR (150MHz, CD_3OD) δ: 126.8(C-1), 130.8 (C-2, C-6), 114.9 (C-3, C-5), 156.4 (C-4), 41.6 (C-7), 175.2 (C-8). These findings were in consensus with previous reports (Zhang *et al*., 2012). Therefore, compound 3 was determined to be p-hydroxyphenylacetic acid.

DISCUSSION

Plant endophytic fungi, an abundant resource, are contained various secondary metabolites such as terpenoids (Zhang *et al*., 2021), alkaloids (Ishiuchi *et al*., 2018) and polyketones (Liu *et al*., 2021), which exhibited enormous potential in plant growth promotion (Baron and Rigobelo, 2021), stress resistance (Fontana *et al*., 2021) and antibacterial (Radic and Strukelj, 2015). Thus, endophytic fungi are very promising microbial resources.



Diversity analysis of endophytic fungi and identification of dominant genera of Dendrobium huoshanense
Fig. 1: The phylogenetic tree for the endophytic fungi based on ITS



Preliminary studies on the secondary metabolites of the dominant genus Stagonosporopsis oculihominis
Fig. 2: The structure of the secondary metabolites of *Stagonosporopsis oculihominis*

Table 1: Diversity and community structure of endophytic fungi from *Dendrobium huoshanense* in different cultivation habitats

Classification		IF (%)							Total
Genus	Species	CL	CM	CS	SL	SM	SS	PM	
Stagonosporopsis	*crystalliniformis*	-	-	-	-	1.2	-	-	17.4
	tanaceti	-	-	2.4	-	-	-	-	
	oculihominis	4.8	3.0	-	-	0.6	-	1.8	
	actaeae	-	-	1.8	-	1.8	-	-	
Pyrenochaeta	*lycopersici*	1.2	-	-	-	-	-	-	3.0
	leptospora	-	-	-	-	1.8	-	-	
Coniothyrium	*multiporum*	0.6	-	-	-	-	-	-	0.6
Septoriella	*phragmitis*	0.6	-	-	1.2	-	-	-	1.8
Alternaria	*eichhorniae*	-	3.6	3.0	3.0	-	4.8	-	19.8
	chartarum	-	-	-	1.8	-	-	-	
	helianthiinficiens	-	2.4	1.2	-	-	-	-	
Pleospora	*herbarum*	-	-	0.6	-	-	-	-	0.6
Fusarium	*ramigenum*	-	2.4	-	-	-	-	-	22.8
	bactridioides	-	-	-	-	1.8	-	1.2	
	guttiforme	-	-	-	-	-	4.2	-	
	concentricum	-	-	-	-	2.4	-	1.2	
	fujikuroi	-	-	-	-	1.2	-	0.6	
	circinatum	-	-	-	2.4	-	-	-	
	lactis	-	-	3.0	-	-	-	2.4	
Phyllosticta	*aristolochiicola*	-	-	2.4	3.0	-	-	-	10.2
	ericarum		3.0	1.8	-	-	-	-	
Botrytis	*caroliniana*	-	-	0.6	-	-	-	-	0.6
Bionectria	*wenpingii*	-	-	0.6	-	-	-	-	0.6
Ulocladium	*dauci*	-	-	-	0.6	-	-	-	0.6
Arthrinium	*hydei*	-	-	-	-	1.2	1.2	-	3.0
	kogelbergense	-	-	-	-	-	0.6	-	
Curvularia	*intermedia*	-	-	-	-	-	0.6	-	0.6
Colletotrichum	*orchidophilum*	-	-	-	-	-	3.0	-	6.6
	brisbanense	-	-	-	-	-	-	0.6	
	gloeosporioides	-	-	-	-	-	3.0	-	
Sordaria	*fimicola*	-	-	-	-	0.6	-	-	0.6
Wojnowiciella	*viburni*	-	-	-	-	0.6	-	-	0.6
Aspergillus	*tubingensis*	-	1.8	-	-	1.2	-	2.4	12.0
	flavus	-	1.8	-	-	1.8	-	2.4	
	ochraceus	-	-	-	-	-	-	0.6	
Acrocalymma	*aquatica*	-	-	-	-	-	-	0.6	0.6
Number of tissues		30	30	30	30	30	30	30	210
Number of contaminated tissues		10	29	28	19	23	28	22	161
Number of strains		12	30	29	20	26	28	23	168
CR		33.33	96.33	93.33	63.33	76.67	93.33	73.33	76.76
IR		40.00	100.00	96.33	66.67	86.67	93.33	76.67	80.00
D		0.8379	1.6760	2.5139	1.3966	3.0726	1.9553	2.5139	13.9662
H		0.2602	0.6538	0.6792	0.4560	0.7135	0.6037	0.5698	3.9362
J		0.1875	0.3384	0.2950	0.2313	0.2787	0.3103	0.2477	1.8889

C, S and P denote the different collection sites; L, M and S denote understorey, sawdust and gravel cultivation, respectively.
"-" indicates that the fungus of this genus/species was not isolated from the plant.

Table 2: Inhibitory effects of metabolites from the five endophytic fungi of *Dendrobium huoshanense* on four bacterial strains

Samples	Concentrations (μg·mL^{-1})	Inhibition rate (%)			
		Escherichia coli	*Staphylococcus aureus*	*Salmonella enterica*	*Pseudomonas aruginosa*
Penicillin G sodium	5		100.181±0.105		
	10			98.538±0.103	
Ceftazidime	2	100.562±0.184			100.369±0.192
Alternaria eichhorniae	128	0.962±2.891	-31.348±2.356	8.385±0.073	-33.211±2.815
Phyllosticta aristolochiicola	128	-3.928±1.837	26.595±1.953	8.64±0.289	-17.288±1.721
Fusarium lactis	128	2.526±2.253	35.079±2.74	7.313±1.155	-35.238±0.83
Stagonosporopsis oculihominis	128	-4.65±1.547	84.898±0.581	5.375±0.145	-24.033±0.939
Aspergillus flavus	128	-7.696±1.89	-17.57±1.532	4.524±1.798	-31.589±0.648

Table 3: Inhibited NO production rates from metabolites of five endophytic fungi of *Dendrobium huoshanense*

Samples	Concentrations	NO inhibition rate (%)
L-NMMA	50 μM	52.94±0.34
Stagonosporopsis oculihominis	50 μg·mL^{-1}	42.99±0.61
Alternaria eichhorniae	50 μg·mL^{-1}	14.89±0.70
Fusarium lactis	50 μg·mL^{-1}	23.41±1.09
Phyllosticta aristolochiicola	50 μg·mL^{-1}	30.44±0.48
Aspergillus flavus	50 μg·mL^{-1}	12.94±1.25

Minimum inhibitory concentrations (mg/mL)

In recent years, the diversity of endophytic fungi of *Dendrobium huoshanense* has been studied thoroughly (Mo *et al*., 2019; Liu *et al*., 2021), but there are few reports on the activity of secondary metabolites, especially the anti-inflammatory and antibacterial activities related to host plants.

Here, in this study, a total of 168 endophytic fungi were isolated from *Dendrobium huoshanense* and their diversity was analyzed and the dominant genera were identified to five strains.

Based on the endosymbiosis theory, we initially examined whether the five dominant strains possessed the same anti-inflammatory and antibacterial activities as their host plant using a solid fermentation model. Our findings suggested that the secondary metabolites of the *Stagonosporopsis oculihominis* strain exerted an inhibitory effect on both *Staphylococcus aureus* and NO release. Moreover, the secondary metabolites of this strain had been reported in our previous research (Yu *et al*., 2017; Liang *et al*., 2018; Wang *et al*., 2021), further their secondary metabolites were traced and three derivatives of benzene ring were initially identified.

The above aromatic derivatives showed the anti-inflammatory activities. For example, compound **1** (PheNA) exhibited significant NO inhibition with IC_{50} values ranging from 2.1 ± 0.3 μM (Liao *et al*., 2017). P-hydroxybenzaldehyde (compound **2**, HD) can effectively reduce TNBS induced Crohn's disease inflammation reaction in mice (Xu *et al*., 2022).

CONCLUSION

These findings indicated that the endophytic fungi of *Dendrobium huoshanense* are diverse and their dominant genera exert similar anti-inflammatory and antibacterial effects as those of the host plant. We initially unraveled the symbiotic relationship between the two and provided a scientific basis for the subsequent investigation of the medicinal value of *Dendrobium huoshanense*.

REFERENCES

Ahlholm JU, Helnader M and Henriksson J (2002). Environmental conditions and host genotype direct genetic diversity of *Venturia ditricha:* A fungal endophyte of birch trees. *Evolution Int. J. Org. Evolution*., **56**(8): 1566-1573.

Ali T, Sarwar A, Anjum AA, Yaqub T, Zeshan B, Ali MA and Sattar MMK (2022). Activity of plant essential oils against antibiotic resistant *Enterococcus faecalis* isolated from diarrheic children. *Pak. J. Pharm. Sci*., **35**(3): 711-719.

Baron NC and Rigobelo EC (2021). Endophytic fungi: A tool for plant growth promotion and sustainable agriculture. *Mycology*, **13**(1): 39-55.

Cai CH, Tan CY and Chen HQ (2020). Chemical constituents from *Dendrobium sinense* (α). *Guihaia*, **40**(9): 1368-1374.

Chen J, Wang H and Guo SX (2012). Isolation and identification of endophytic and mycorrhizal fungi from seeds and roots of *Dendrobium* (Orchidaceae). *Mycorrhiza*, **22**(2): 297-307.

Debary A (1866). Morphologie and physiologie derpilze. Flenchten and Myxomyceten. Engelmann Leipzig., **4**(1): 1-316.

Fisher PJ and Petrini O (1992). The distribution of some fungal and bacterial endophytes in maize. *New. Phytol*., **122**(2): 299-305.

Fontana DC, de Paula S, Torres AG, de Souza VHM, Pascholati SF, Schmidt D and Neto DD (2021). Endophytic fungi: Biological control and induced resistance to phytopathogens and abiotic stresses. *Pathogens*, **10**(5): 570.

Ishiuchi K, Hirose D, Suzuki T, Nakayama W, Jiang WP, Monthakantirat O, Wu JB, Kitanaka S and Makino T (2018). Identification of lycopodium alkaloids produced by an ultraviolet-irradiated strain of paraboeremia, an endophytic fungus from *Lycopodium serratum* var. longipetiolatum. *J. Nat. Prod*., **81**(5): 1143-1147.

Laksmitawati DR, Widyastuti A, Karami N, Afifah E, Rihibiha DD, Nufus H and Widowati W (2017). Anti-inflammatory effects of Anredera cordifolia and Piper crocatum extracts on lipopolysaccharide-stimulated macrophage cell line. *Bangladesh J. Pharmacol*., **12**(1): 35-40.

Liang YM, Yu Y, Sun YP, Liu JS. Ma ZH, Liu HT and Wang G (2018). Secondary metabolites of endophytic fungus *Stagonosporopsis oculihominis* from *Dendrobium huoshanense*. *Nat. Prod. Res. Dev.*, **30**(5): 783-788.

Liao YR, Kuo PC and TsaI WJ (2017). Bioactive chemical constituents from the root bark of *Morus australis*. *Bio. Med. Chem. Lett.*, **27**(2): 309-313.

Liu SZ, He FM, Bin YL, Li CF, Xie BY, Tang XX and Qiu YK (2021). Bioactive compounds derived from the marine-derived fungus MCCC3A00951 and their influenza neuraminidase inhibition activity *in vitro* and *in silico*. *Nat. Prod*. Res., **35**(24): 5621-5628

Liu L, Chen ST, Zhang T, Li L, Liu XY and Han BX (2021). Diversity and difference of mycorrhizal fungi in *Dendrobium huoshanense*. *Nat. Prod. Res. Dev.*, **33**(11): 1894-1900.

Liu S, Zhao YP, Heering C, Janiak C, Muller WEG, Akone SH, Liu Z and Proksch P (2019). Sesquiterpenoids from the Endophytic Fungus *Rhinocladiella similis*. *J. Nat. Prod*., **82**(5): 1055-1062.

Mo QG, Fu HY, Zhou G and Wang YL (2019). Research progresses of endophytic fungi from *Dendrobium huoshanense* and its secondary metabolite. *J. Wuhan Univ. (Nat. Sci. Ed.).*, **65**(4): 333-339.

Petrini O (1991). Fungal endophytes of tree leaves [A]. *In*: Microbial Ecology of Leaves (eds. Andrews JH, Hirano SS). Spring-Verlag, New York, USA, **3**(2): 179-197.

Qi HX, Jia Q and Gao Y (2015). Diversity and distribution of endophytic fungi from *Lycium barbarum* L. of Ningxia. *Nor. Horticul*., **44**(13): 153-157.

Radic N and Strukelj B (2015). Endophytic fungi-the treasure chest of antibacterial substances. *Phytomedicine*, **19**(14): 1270-1284.

Ratnaweera PB, Walgama RC, Jayasundera KU, Herath SD, Abira S, Williams DE, Andersen RJ and de Silva ED (2018). Antibacterial activities of endophytic fungi isolated from six Sri Lankan plants of the family Cyperaceae. *Bangladesh J. Pharmacol*. **13**(3): 264-272.

Ren FC, Liu L, Lv Y.F, Bai X, Kang QJ, Hu XJ, Zhuang HD, Liu Y, Hu JM and Zhou J (2021) Antibacterial prenylated p-hydroxybenzoic acid derivatives from *Oberonia myosurus* and identification of putative prenyltransferases. *J. Nat. Prod*., **84**(2): 417-426.

Su SQ, Jiang H, Li QM, Zha XQ and Luo JG (2020). Study on chemical constituents of *Dendrobium huoshanense* stems and their anti-inflammatory activity. *Chin. Chin. Mater. Med.*, **45**(14): 3452-3458.

Stierle A and Stierle D (1993). Taxol and taxane production by Taxomyces andreanae: An endophytic fungus of Pacific yew. *Science*, **260**(5105): 214-216.

Wang D, Jia SH, Zhang ZX, Cai YP and Lin Y (2007). Isolation and culture of an endophytic fungus associated with *Dendrobium huoshanense* and its effects on the growth of plantlets. *J. Fung. Res*., **5**(2): 85-88.

Wang JT, Li HY, Rao R, Yue JY, Wang GK and Yu Y (2021). (±)-Stagonosporopsin A, stagonosporopsin B and stagonosporopsin C, antibacterial metabolites produced by endophytic fungus *stagonosporopsis oculihominis*. *Phytochem. Lett*., **45**(6): 157-160.

Waqar-ul-Konain M, Zeenat H and Idrees M (2022). Datura innoxia antimicrobial activities against *E. coli* isolated from infections of type 2 diabetic patients. *Pak. J. Pharm. Sci*., **35**(2): 479-486.

White JFJr, Morrow AC, Morgan-Jones G and Chambless DA (1996). Endophyte hostassociations in forage grasses. XIV. Primary storaata formation and seed transmission in *Epichloe typhina*: developmental and regulatory aspect. *Mycologia*, **83**(1): 72-81.

Xu XF, Luo AL, Xu XT, Lu X, Wang YH and Duan XQ (2022). Study of the protective effect of p-hydroxybenzaldehyde against TNBS-induced Crohn's disease in mice based on PPARγ/AMPK/NF-κB signaling pathway. *Nat. Prod. Res. Dev.*, **34**(7): 1-11.

Yu M, Ran HL, Mei WL, Huang SZ, Wei YM, Cai CH, Dai HF and Wang H (2022). Study on chemical constituents and their anti-inflammatory activity from stems of *Strophanthus divaricatus*. *Nat. Prod. Res. Dev.*, **34**(1): 57-62.

Yu Y, Ma BJ, Liu JS, Yue JY, Chen HP, Liang YM, Zhou ZY, Wang GK and Wang G (2017). Two new alkaloid metabolites produced by endophytic fungus *stagonosporopsis oculihominis* isolated from *Dendrobium huoshanense*. *Phytochem. Lett.*, **19**(1): 266-270

Zang W, Sun X and Sun JQ (2014). Diversity and community structure of endophytic fungi from *Taxus chinensis* var. Mairei. *Chin. J. App. Eco*., **25**(7): 2071-

2078.

Zhang M, Xu L X and Xue JH (2012). Metabolits of *Fusarium solani*. *J. Trop. Subtrop. Bot*., **20**(6): 585-590.

Zhang WG, Lu XX, Huo LQ, Zhang S, Chen Y, Zou ZX and Tan HB (2021). Sesquiterpenes and steroids from an endophytic *Eutypella scoparia*. *J. Nat. Prod*., **84**(6): 1715-1724.

Zhao HS, Xu FQ, Chen XX, Hu JM, Zeng FJ, Peng DY and Wu DL (2021). Chemical constituents of *Dendrobium huoshanense* C. Z. Tang et S. J. Cheng. *Nat. Prod. Res. Dev.*, **33**(9): 1491-1498.