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Fusarium fujikuroi WQF5 ISOLATED FROM *Cephalotaxus mannii* Hook.f. AS A PRODUCER OF ANTIBACTERIAL AGENT AND PACLITAXEL

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ABSTRACT

Cephalotaxus mannii Hook.f is a rare medicinal plant used for the treatment of leukemia, however, its fungal endophytes with antibacterial and anticancer properties have not been exploited yet. In this study, six fungal isolates were recovered from roots of *C. mannii* collected in Ha Giang province, Vietnam. Among them, ethyl acetate crude extract of strain WQF5 exhibited strong antibacterial activity against 5 tested pathogens with inhibition zones ranging from 13.0 ± 0.5 mm to 20.0 ± 0.4 mm. Sulforhodamine B assay showed that WQF5 extract possessed the most potent cytotoxic activity against lung cancer A549 and breast cancer MCF7 cell lines with IC_{50} values of 6.9 ± 0.7 µg/mL and 23.1 ± 1.9 µg/mL, respectively. PCR-based molecular marker screening revealed that the positive hits for essential genes encoding 10-deacetylbaccatin III-10-O-acetyltransferase (*dbat*) and C-13 phenylpropanoyl side chain-CoA acyltransferase (*bapt*) involved in paclitaxel production were found in the fungal isolate WQF5. In addition, isolate WQF5, identified as *Fusarium fujikuroi* by morphological and ITS analysis, also produced paclitaxel as shown by HPLC-DAD analysis. This is the first report of bioprospecting endophytic fungi isolated from *C. mannii*, in which the capability of producing paclitaxel of endophytic fungi was also proved for the first time. These findings addressed a potent candidate for paclitaxel production and provided excellent material for further investigations of how endophytic fungi from non-*Taxus* plant species synthesize paclitaxel.

Keywords: Anticancer, *bapt*, *Cephalotaxus mannii*, *dbat*, *Fusarium fujikuroi*, paclitaxel.

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INTRODUCTION

Endophytic fungi are known to colonize inside plant tissues without inflicting negative effects and even promote the ecological adaptability of their host under every environmental condition. Especially, fungal endophytes can mimic secondary metabolites from the host due to microbe-host interactions, which are an important source for the pharmaceutical industry (Zhou et al., 2007; Vu et al., 2022). The billion-dollar anticancer drug paclitaxel is widely used for the treatment of various cancers, which has only been extracted from the bark of *Taxus* species such as *Taxus* x *media* and *Taxus baccata* being listed in the International Union for Conservation of Nature (IUCN) Red List (Garyali et al., 2013). The increasing demand for paclitaxel and the shortage of plant resources have led to a growing interest in exploiting an alternative source such as endophytic fungi. To date, more than 20 genera of fungal endophytes associated with *Taxus* species are able to produce paclitaxel and other taxanes (Yang et al., 2014). In contrast to the horizontal gene transfer hypothesis accepted by many scientists, paclitaxel can be found in non-*Taxus* species such as *Ginko biloba* and *Corchorus olitorius* (Das et al., 2017; Abdel-Fatah et al., 2021). It further promotes the need to discover paclitaxel biosynthesis genes in fungal endophytes and their evolutionary origin.

Since paclitaxel detection of culturable fungi is laborious and time-consuming, a PCR-based molecular marker is supposed to be an effective method to screen paclitaxel-producing fungi. Genes coding for taxadiene synthase (*ts*), 10-deacetylbaccatin III-10-O-acetyltransferase (*dbat*), and C-13 phenylpropanoyl side chain-CoA acyltransferase (*bapt*) were utilized as molecular markers to identify endophytic fungi capable of producing paclitaxel (Das et al., 2017). Protein TS contributes to the formation of the taxane skeleton as the first step of paclitaxel biosynthesis. DBAT is involved in the synthesis of baccatin III, an immediate diterpenoid precursor of paclitaxel. In the next step, β-phenylalanoyl baccatin III, a precursor of paclitaxel is synthesized by BAPT, which highly confirms paclitaxel production by endophytic fungi.

Besides the production of plant-derived metabolites, endophytic fungi are also a potent source of antimicrobial compounds. Endophytic-fungus *Pestalotiopsis mangiferae* from *Mangifera indica* produced 4-(2,4,7-trioxa-bicyclo [4.1.0] heptan-3-yl) phenol that was highly active against *Bacillus subtilis*, *Klebsiella pneumoniae*, *Escherichia coli*, *Micrococcus luteus*, and *Pseudomonas aeruginosa* (Subban et al., 2013). Novel compounds fusapyridon A, rhein, and fusaric acid from endophytic *Fusarium* spp. exhibited potent inhibitory effects on bacterial pathogens (Deshmukh et al., 2015). In addition, genomes of endophytic fungi consisted of a large number of secondary metabolite biosynthetic gene clusters in which various gene clusters were inactivated under laboratory cultivation conditions (Wiemann et al., 2013). The possibility of discovering new antibacterial agents is still being considered by scientists and remains promising.

Cephalotaxus mannii Hook.f. is a coniferous plant species classified as vulnerable in the IUCN Red List distributed in China and Southeast Asia such as Vietnam (Saithong et al., 2010; Van Loc et al., 2017). *C. mannii* is used for the treatment of leukemia due to the presence of bioactive compounds such as cephalotaxine, isoharringtonine, norisoharringtonine, desoxyharringtonine, nordesoxyharringtonine, cephalotaxine *β*-N-oxide, 3-epi-schellhammericine, and cephalezomine (Van Loc et al., 2017). Earlier work only reported the isolation of endophytic fungi from *C. mannii* collected in Thailand and China (Saithong et al., 2010). The present study aimed to screen the antibacterial and anticancer properties of endophytic fungi recovered from roots of *C. mannii* collected in Ha Giang province, Vietnam. In addition, a new paclitaxel-producing fungus, identified as *Fusarium fujikuroi* WQF5, was also identified using PCR-based molecular markers and

HPLC-DAD. To our knowledge, this is the first report proving that paclitaxel can be produced by endophytic fungi associated with *C. mannii*.

MATERIALS AND METHODS

Isolation of endophytic fungi

The roots of *Cephalotaxus mannii* Hook.f. were collected in the Bat Dai Son Nature Reserve (23º8'16"N-105º0'44"E; 1,230 meters in height), Ha Giang province, Vietnam. The root samples were transported to the laboratory of the Institute of Biotechnology, Vietnam Academy of Science and Technology for isolation of endophytic fungi. Leaves and roots were sent to the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology for plant identification. All samples were surface sterilized, homogenized, and then inoculated on potato dextrose agar (PDA) supplemented with 100 mg/L streptomycin (Kumar et al., 2019). Once grown onto PDA plates, a single hypha was carefully subcultured onto fresh PDA plates. For pure isolates, spores and mycelia of each fungal strain were preserved in 15% (v/v) glycerol at -80 ºC.

Antimicrobial assay

Six fungal endophyte samples were cultivated separately, each sample was in 500 mL of fresh potato dextrose broth (PDB) at 28 ºC on a rotary shaker for 14 days. Each culture was filtered by vacuum filtration and extracted twice with three volumes of ethyl acetate in a separatory funnel. After that, a solvent phase was concentrated and evaporated to dryness using a vacuum rotary evaporator at 45 ºC (Vu et al., 2022). Each crude extract was weighed and dissolved in 1% (v/v) dimethyl sulfoxide (DMSO).

To evaluate the antimicrobial property of obtained fungi, the agar-well diffusion method was performed with a slight modification (Quach et al., 2022). Bacterial pathogens used in this study included: two Gram-negative bacteria (*Escherichia coli* ATCC 11105, *Pseudomonas auroginosa* ATCC 9027), four Gram-positive bacteria (*Bacillus cereus* ATCC 11778, methicillin-resistant *Staphylococcus epidermidis* (MRSE) ATCC 35984, methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591 and *Enterococcus faecalis* ATCC 29212), and one yeast *Candida albicans* ATCC 10231. Erythromycin and nystatin were the positive controls for bacteria and fungi respectively, while 1% (v/v) DMSO used to resuspend fungal extracts was included as negative controls.

Cytotoxic assay

All fungal crude extracts were used to assess the cytotoxicity on human lung cancer A549 and human breast MCF7 cell lines as described previously (Skehan et al., 1990). Cell lines were plated in 96-well microtiter plates at a density of 10^4 cells/well for 24 hours, which were subsequently treated with the fungal crude extract at different concentrations and left for 72 hours. Then, the cells were fixed with 20% (w/v) trichloroacetic acid for an hour, stained with 0.4% (w/v) sulforhodamine B (SRB) at 37 ºC for 30 minutes, and rinsed 3 times with acetic acid before drying out at room temperature. The optical density of the reaction was measured at 540 nm by a microplate reader (BioTek EXL800). The IC_{50} value, which was the concentration at which samples inhibit 50% of cancer cells in comparison with the control grown in the same conditions, was computed by the TableCurve 2Dv4 program. About 10 µg/mL ellipticine and 1% (v/v) DMSO were used as positive and negative controls, respectively.

Morphological and molecular identification of fungal strain WQF5

The fungal strain WQF5 was cultivated on a PDA medium at 30 ºC to observe the growth rate of mycelium and colony morphology. The morphology of conidiophores and conidia was observed under a light microscope at 40X (Olympus, Japan).

The genomic DNA of the fungus was extracted using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany). The internal

transcribed spacer (ITS) sequence of WQF5 was amplified using the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Ngo et al., 2021). The PCR reaction mixtures contained 1 µl genomic DNA (~300 ng), 0.5 µL forward and reverse primers (20 µM), 12.5 µL PCR Master Mix (Promega), and 10.5 µL PCR water. The PCR reaction cycling parameters included pre-heating at 94 ºC for 5 min, 30 cycles of 94 ºC for 1 min, 55 ºC for the 30 s, 72 ºC for 1 min, and a final extension at 72 ºC for 10 min. The PCR products were visualized on 1% (w/v) agarose gel and purified using The Wizard™ SV Gel and PCR Clean-Up System (Promega), before being sent for sequencing on a Sanger sequencer by First BASE Laboratories Sdn. Bhd. (Malaysia). Sequences were processed using BioEdit software (ver. 7.2.5, USA) and compared with the NCBI database (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov) using the BLAST tool. A phylogenetic tree was built using Mega software v.11 by the neighbor-joining method (Kumar et al., 2016).

Primary screening of paclitaxel-producing fungi based on PCR amplification

Given that *ts*, *dbat*, and *bapt* genes essential for paclitaxel biosynthetic pathway are conserved across *Taxus* species and their endophytic fungi, specific primers *dbat*-F (5'-ATGGCTGACACTGACCTCTCAGT-3'), *dbat*-R (5'-GGCCTGCTCCTAGTCCATCACAT-3'), *bapt*-F (5'-CCTCTCTCCGCCATTGACAACAT-3'), *bapt*-R (5'-GTCGCTGTCAGCCATGGCTT-3') *ts*-F (5'-ATCAGTCCGTCTGCATACGACA-3'), *ts*-R (5'-TAAGCCTGGCTTCCCGTGTTGT-3') were synthesized followed previous studies (Zhou et al., 2007; Kumar et al., 2019). PCR amplification steps for these three genes were similar to the process of amplification of the ITS gene described above. The PCR products of *ts*, *dbat*, and *bapt* genes were analyzed in 2% (w/v) agarose gel.

Determination of paclitaxel-producing fungi

Determination of the paclitaxel present in the 10 mg/mL of crude extract WQF5 was carried out using HPLC analysis using an Agilent 1200 HPLC system equipped with a diode array detector (DAD) (Agilent Technologies, Palo Alto, CA, USA) with some modifications (El-Bialy & El-Bastawisy, 2020). Briefly, crude extracts were reconstituted in acetonitrile and subjected to an Eclipse XDB-18 the reverse-phase column Eclipse XDB-18 (Agilent Technologies, USA) at a temperature from 25.3 to 26.7 ºC. The sample was eluted with gradient acetonitrile and deionized water as a mobile phase and the flow rate was 0.5 mL/min. Ultraviolet detection was performed at the wavelength from 210 nm to 365 nm. The standard paclitaxel (Sigma) dissolved in methanol at a concentration of 1 mg/mL was employed for comparison. The presence of paclitaxel in fungal extract WQF5 was determined by comparison to retention times (Rt) and UV spectra of the standard paclitaxel.

RESULTS AND DISCUSSION

Isolation and screening of antimicrobial properties of fungal endophytes

A total of six endophytic fungi samples with different morphological characteristics were isolated from the root samples of *C. mannii* using PDA. After that, the ethyl acetate crude extracts from six fungal isolates were evaluated for their antimicrobial activity against seven human pathogens. It revealed that four out of six crude extracts (66.7%) were active against at least one pathogen with an inhibition zone ranging from 4.3 ± 0.5 to 29.7 ± 1.5 mm (Fig. 1). None of those extracts showed inhibition against *C. albicans* ATCC 10321. Of note, WQF5 showed significant inhibitory effects on *E. coli* ATCC 11105 (13.0 ± 0.7 mm), *P. aeruginosa* ATCC 9027 (18.0 ± 0.4 mm), MRSE ATCC 35984 (20.0 ± 0.4 mm), MRSA ATCC 33591 (15.0 ± 0.7 mm), and *E. faecalis* ATCC 29212 (19.0 ± 0.4 mm). At the lower level, WQF3 was only active against four bacterial pathogens such as

P. aeruginosa ATCC 9027, *B. cereus* ATCC 11778, MRSA ATCC 33591, and *E. faecalis* ATCC 29212 with inhibition zones ranging from 10.4 ± 0.4 to 13.4 ± 0.8 mm. The percentage of bioactive fungi with antibacterial properties from the roots of *C. mannii* was higher than those in previous reports in similar studies. For example, only 33.6% of endophytic fungi from the leaves of the medicinal plant *Indigofera suffruticosa* Miller showed antibacterial activity (Santos et al., 2015). Only 8% of fungal strains recovered from *Chloranthus japonicus* Sieb exhibited inhibitory activities against at least one tested pathogen (An et al., 2020). These results highlight the potential of producing bioactive compounds with antibacterial activity by fungal endophytes from *C. mannii.*

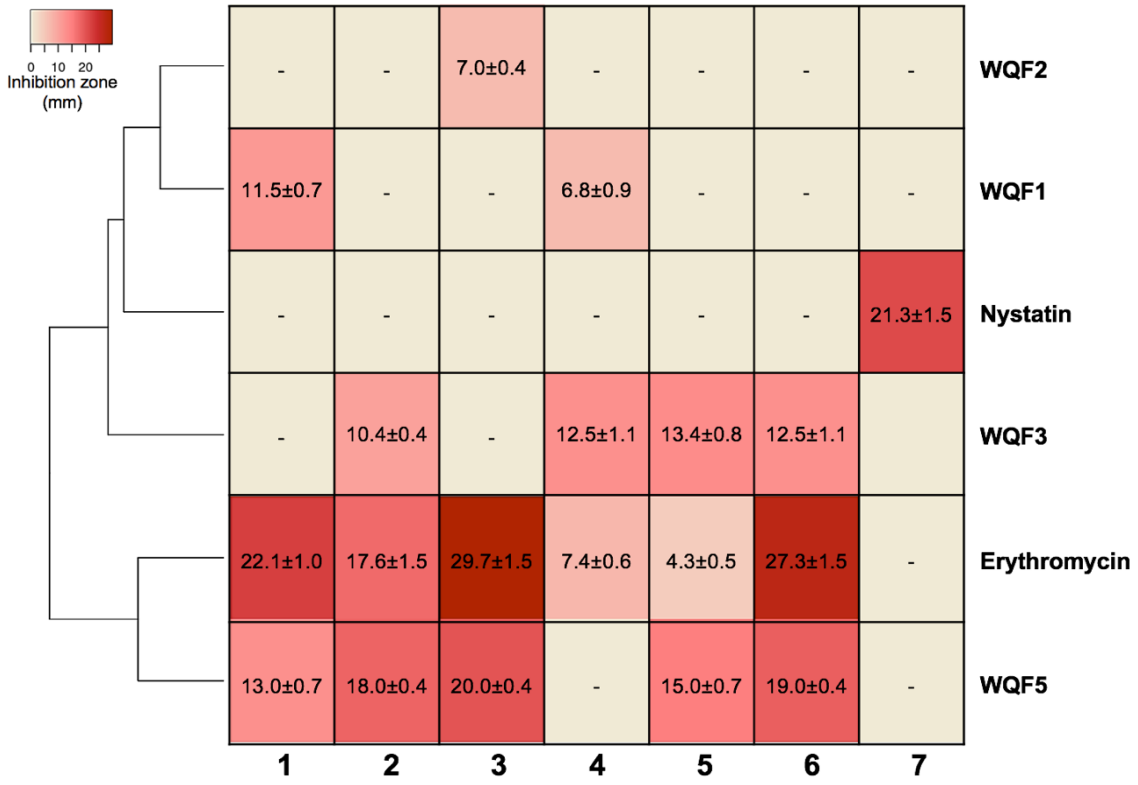

Figure 1. Heatmap revealing antimicrobial property of endophytic fungi isolated from *Cephalotaxus mannii*. (1) *Escherichia coli* ATCC 11105, (2) *Pseudomonas aeruginosa* ATCC 9027; (3) MRSE ATCC 35984; (4) *Bacillus cereus* ATCC 11778; (5) MRSA ATCC 33591; (6) *Enterococcus faecalis* ATCC 29212, and (7) *Candida albicans* ATCC 10321

Cytotoxic potential screening of fungal endophytes

The cytotoxic potential of fungal extracts to inhibit A549 and MCF7 cancer cell growth was determined using the SRB assay. All extracts showed no cytotoxic activity against A549 and MCF7 cancer cell lines, except for WQF5. Indeed, treatment of A549 and MCF7 cells with the fungal extract WQF5 resulted in remarkable inhibition with IC_{50} values of 6.9 ± 0.7 µg/mL and 23.1 ± 1.9 µg/mL, respectively (Table 1). It is similar to our previous study demonstrating that only 4/16 fungal extracts from *Tsuga chinensis* (Franch.) Pritz. in Ha Giang province showed cytotoxicity on A549 and MCF7 cells, among them the most interesting strain *Penicillium* sp. SDF5 inhibited A549 and MCF7 cell lines with

IC_{50} values of 14.2 ± 1.5 µg/mL and 25.2 ± 1.7 µg/mL, respectively (Vu et al., 2022). The United States National Cancer Institute Plant Screening Program claims that a crude extract has *in vitro* potent cytotoxicity if the IC_{50} value is lower than 30 µg/mL (Majoumouo et al., 2020), suggesting that the presence of bioactive compounds within WQF5 extract are potential for development as anticancer agents.

Table 1. Cytotoxic activity and PCR analysis for the presence of paclitaxel biosynthetic genes of endophytic fungi from *Cephalotaxus mannii*

Strain	Cytotoxity IC_{50} (µg/mL)		Paclitaxel biosynthetic genes		
	A549	MCF7	*dbat*	*bapt*	*ts*
WQF1	> 100	> 100	+	-	-
WQF2	> 100	> 100	+	-	-
WQF3	> 100	> 100	-	-	+
WQF4	> 100	> 100	-	-	-
WQF5	6.9 ± 0.7	23.1 ± 1.9	+	+	-
WQF6	> 100	> 100	-	-	-
Ellipticine	0.4 ± 0.03	0.6 ± 0.08			

Note: (-): Negative hit; (+): positive hit.

To further evaluate the cytotoxicity of fungal endophytes from *C. mannii*, the conserved sequences of the main genes responsible for paclitaxel biosynthesis including *dbat*, *bapt* and *ts* were studied using PCR. After PCR amplification, fungal isolates WQF1, WQF2**,** and WQF5 showed a positive hit for *dbat* gene with an amplification length of around 550 bp (Table 1). Fungal isolates WQF3 and WQF5 showed amplification of the 600 bp and 500 bp fragments of *ts* and *bapt*, respectively. Of note, the presence of both *dbat* and *bapt* was observed in the isolate WQF5. Thus, isolate WQF5 was selected for further investigations.

Given that identification of paclitaxel-producing fungi is labour-consuming, the use of a molecular marker is an effective tool to obtain potent candidates. The rate-limiting enzyme encoded by the *ts* gene is involved in constructing a unique taxane skeleton as the first step in paclitaxel biosynthesis (Zhou et al., 2007), which does not confirm the production of fungal paclitaxel because more than 10 enzymatic reactions take place after this step. In contrast, both *dbat* and *bapt* are more diagnostic. Moreover, a previous study showed that the positive hits for *ts* and *dbat* were not subjected to paclitaxel production due to the fact that some fungal strains only produce baccatin III instead of paclitaxel (Garyali et al., 2013). It holds true that fungal isolate WQF5 from *C. mannii* likely produces paclitaxel with the presence of both *dbat* and *bapt*. Despite being hypothesized to be the result of horizontal gene transfer, paclitaxel-producing fungi have also been exploited in a few medicinal plants such as *Ginkgo biloba* and *Terminalia arjuna* (Abdel-Fatah et al., 2021). It is possible that the paclitaxel biosynthetic pathway in fungal endophytes is complicated and has a different evolutionary pattern as compared to *Taxus* plants. Hence, this is the first report proving that paclitaxel can be produced by endophytic fungi associated with *Cephalotaxus.*

Identification of the bioactive strain WQF5

Morphological identification revealed that fungal isolate WQF5 thrived on the PDA plates with colony diameters ranging from 2.1 cm to 2.8 cm (Fig. 2A). Fungal colony was white cottony and pale orange undersurface on PDA. The microconidia were oval, elongated oval, pointed ends, and separated into 3–8 (Fig. 2B). Chlamydospores were relatively abundant in the mycelium and appeared subglobose,

intercalary, and rough-walled. Fungal isolate WQF5 morphology was most similar to what has been described as *Fusarium fujikuroi* (Ibrahim et al., 2016).

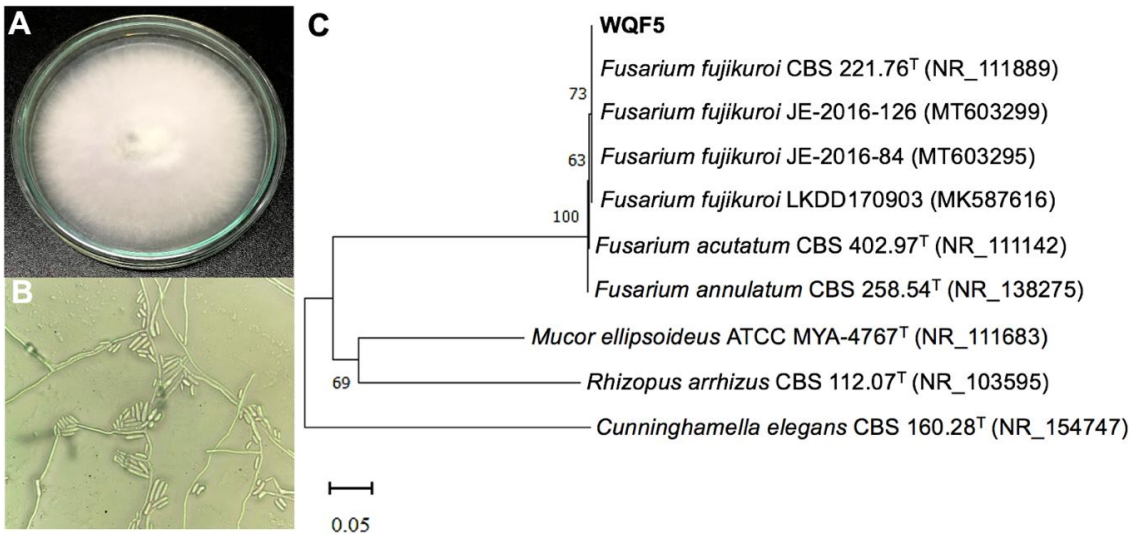

Figure 2. Morphological characteristics (A and B) and phylogenetic tree (C) based on the ITS gene sequences of the strain WQF5 with closely related fungal strains

To strengthen morphological identification results, molecular identification using ITS gene analysis was carried out. PCR amplification with a pair of ITS primers gave a sharp and single band of approximately 550 bp, which was subsequently sequenced. BLAST analysis revealed that the ITS sequence of WQF5 displayed 100% and 99.4% similarity with *F. fujikuroi* CBS 221.76^T and *Fusarium acutatum* CBS 402.97^T, respectively. In addition, the phylogenetic analysis also clustered WQF5 with 4 *F. fujikuroi* species (Fig. 2C). The ITS sequence of WQF5 was deposited at GenBank (NCBI) under accession number OP482181. Based on morphological and molecular identification, WQF5 was identified as *F. fujikuroi* WQF5.

F. fujikuroi is known to cause bakanae disease epidemic of rice previously reported in Japan (Chen et al., 2020). In another aspect, *F. fujikuroi* is an industrial fungi used for the production of gibberellic acids, a group of extensive phytohormones (Cen et al., 2020). Extraction of secondary metabolites of *F. fujikuroi* led to the identification of alkaloid 2-(4-butylpicolinamide) acetic acid, terpestacin, and fusaric acid, among them only fusaric acid was moderately active against *S. aureus*, *E. coli*, and *Salmonella setubal* (Hilário et al., 2016). Given that 45 putative gene clusters were reported in *F. fujikuroi* using whole-genome sequencing (Wiemann et al., 2013), the possibility of exploiting new secondary metabolites with antibacterial and anticancer activity is still promising.

Determination of paclitaxel in the extract of *Fusarium fujikuroi* WQF5

The crude WQF5 extract that was active against 2 cancer cell lines was tested for the presence of paclitaxel using HPLC analysis. The retention time (Rt) of standard paclitaxel (Sigma) was 35.875 min (Fig. 3A), while the chromatogram of WQF5 exhibited a peak with Rt at 35.806 min (Fig. 3C). Moreover, the UV spectra of WQF5 extract matched that of the standard paclitaxel (Figs. 3B, 3D). These results confirmed the presence of paclitaxel in the *F. fujikuroi* WQF5 extract.

To the best of our knowledge, this is the first report of endophytic *F. fujikuroi*

capable of producing paclitaxel isolated from non-*Taxus* plant. *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Monochaetia*, *Pestlotia*, *Pestalotiopsis*, *Pithomyces*, *Penicillium* and *Xylaria* have been reported as paclitaxel-producing fungi derived from yew trees. Among 32 *Fusarium* species, only *Fusarium redolens*, *Fusarium solani*, and *Fusarium oxysporum* isolated from *Taxus* plants were reported to produce paclitaxel (Chakravarthi et al., 2008; Elavarasi et al., 2012; Garyali et al., 2013). In addition, using genome sequencing, *Penicillium aurantiogriseum* NRRL 62431 was shown to evolve independently to synthesize paclitaxel due to the presence of 7 potential homologs to 13 known paclitaxel biosynthetic genes from *Taxus* (Yang et al., 2014). In line with that, horizontal gene transfer between this endophytic fungus and its host plant is unlikely. Regarding non-*Taxus* plant such as *C. mannii*, the ability of some fungal endophytes to synthesize paclitaxel remains an unanswered question. Thus, whole-genome sequencing and genome mining of *F. fujikuroi* WQF5 will be interesting subjects for future studies.

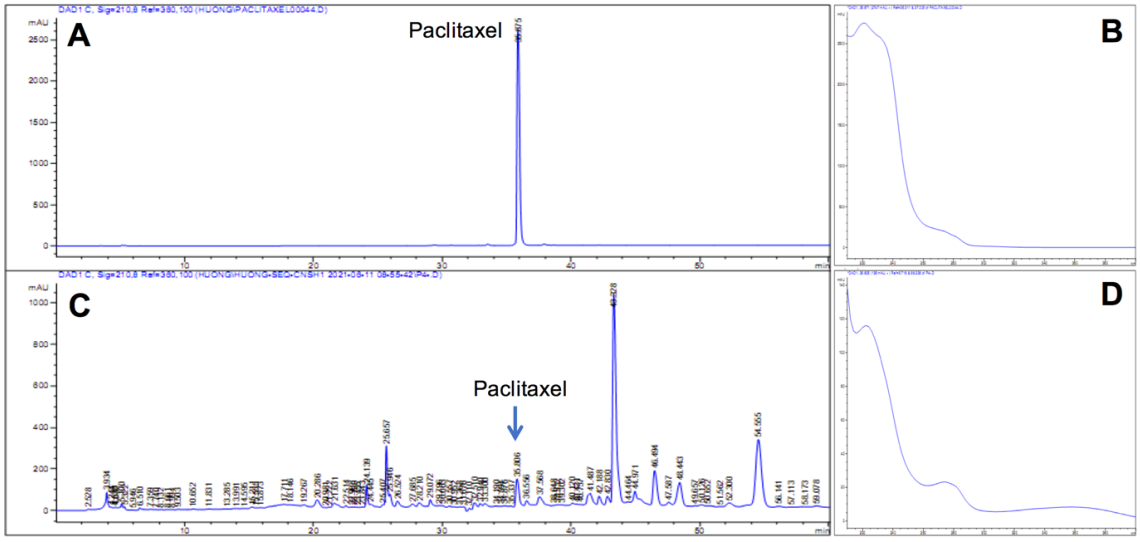

Figure 3. Determination of paclitaxel using HPLC-DAD. Chromatogram (A) and UV spectrum (B) of the standard paclitaxel (Sigma); Chromatogram (C) and UV spectrum (D) of *Fusarium fujikuroi* WQF5 extract

CONCLUSION

In the present study, six endophytic fungi were isolated from the roots of *C. mannii* that is considered vulnerable in the IUCN Red List. Among them, *F. fujikuroi* WQF5 possessed not only strong antibacterial activity against 5 tested pathogens but also cytotoxic activity against A549 and MCF7 cell lines. Moreover, *F. fujikuroi* WQF5 was found to produce paclitaxel through PCR-based screening and HPLC analysis. This is the first report proving the production of paclitaxel by *F. fujikuroi* isolated from a non-*Taxus* plant. Further studies are required to sequence the whole genome of *F. fujikuroi* to shed new light on how *F. fujikuroi* synthesizes paclitaxel.

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