Vietnam Journal of Biotechnology **23**(2): 205-215, 2025. DOI: 10.15625/vjbt-21902

IDENTIFICATION AND CHARACTERIZATION OF ENDOPHYTIC FUNGI ISOLATED FROM *Ixora chinensis* AS POTENT SOURCE OF ANTIBACTERIAL AND ANTICANCER AGENTS

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Received: 05.08.2024
Accepted: 09.12.2024

ABSTRACT

Endophytic fungi isolated from medicinal plants are known as prolific sources of bioactive compounds for medicinal exploitation. The present study focused on the evaluation of the distribution of fungal endophytes from *Ixora chinensis* collected in Ba Vi National Park and their antibacterial and anticancer activities. A total of 14 endophytic fungi were isolated from *I. chinensis*, among them, the highest number of endophytic fungal isolates were found in leaves (28.6%), followed by branches (21.4%), roots (21.4%), flowers (14.3%), and stems (14.3%). A combination of ITS gene sequence and morphological analysis indicates that they belonged to 8 genera, including *Phyllosticta, Diaporthe, Neopestalotiopsis, Botryosphaeria, Pestalotiopsis, Lasiodiplodia, Talaromyces*, and *Colletotrichum.* Among them, *Diaporthe tectonae* IBV6R1 was found to show the strongest inhibitory effect on A549 cells with the percentage inhibition of 71.8 ± 2.3% and antibacterial activity against *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 11105, and *Staphylococcus aureus* ATCC 25923 with the inhibition zones ranging from 1.5 - 21.6 mm. Further analysis also revealed that *D. tectonae* IBV6R1 produced 115.6 µg/L camptothecin using HPLC analysis. This is the report demonstrating the antibacterial and anticancer properties of *D. tectonae*. These findings recommend that *D. tectonae* IBV6R1 may aid in the development of novel compounds and fungal camptothecin. Future investigations are required to purify and elucidate antibacterial compounds as well as optimize camptothecin productivity.

Keywords: Antibacterial activity, camptothecin, *Diaporthe tectonae,* endophytic fungi*, Ixora chinensis*

INTRODUCTION

Fungal endophytes are known to colonize plant tissues successfully without any negative effects on the host as well as secrete bioactive metabolites to enhance antagonism against phytopathogens and plant growth (Vu *et al.*, 2022; Wu *et al.*, 2022). In the last

decades, a number of bioactive metabolites with cytotoxic, antimicrobial, and antioxidant activities have been successfully extracted from fungal endophytes (Vu *et al.*, 2024; Xu *et al.*, 2021). *Diaporthe* sp. LG23 produced ergosterol derivatives, which showed strong inhibition against *Escherichia coli* DSM 682 and *Pseudomonas aeruginosa* DSM 22644 (Silva Cardoso & Macedo, 2022). *Pestalotiopsis karstenii* recovered from *Camellia sasanqua* was found to synthesize pestalrone B that exhibits remarkable cytotoxic activity against HeLa, HepG2, and U-251 (Luo du *et al.*, 2012). Recently, a new bisabolane-type sesquiterpene, namely fusafoetriol, was isolated from *Fusarium foetens* AQF6 colonizing on *Amentotaxus yunnanensis* (Vu *et al.*, 2024).

Interestingly, fungal endophytes have been shown to produce various bioactive metabolites that are identical to their host plants. Camptothecin extracted from plants is regarded as the commercial drug used for cancer treatment (Eldeghidy *et al.*, 2023). Camptothecin was first reported to be produced by the endophytic fungus *Entrophospora infrequens* (Eldeghidy *et al.*, 2023), which promotes various investigations for exploring camptothecin-producing fungi to date. Similar to camptothecin, the anticancer drug paclitaxel is extracted not only from the Pacific yew tree, *Taxus brevifolia*, but also from the endophytic fungus *Fusarium solani* PQF9 (Vu *et al.*, 2023). *Huperzia javanica* was the host of 9 endophytic fungi that produce acetylcholinesterase inhibitors, huperzine A and B (Le *et al.*, 2024). Hence, the search for new endophytic fungi capable of producing plant compounds continues to attract more attention from scientists around the world. *Ixora chinensis* is a tropical evergreen shrub found throughout Vietnam. The flowers, leaves, roots, and stem are used in the Vietnamese traditional system of medicine. In the previous study, *I. chinensis* collected in Bac Giang province is the host of endophytic fungi capable of producing the anticancer drug, camptothecin (Doan *et al.*, 2024). However, other biological activities were not evaluated in our previous work. Therefore, the present study collected *I. chinensis* grown in Ba Vi National Park to explore the diversity of culturable endophytic fungi and demonstrate that endophytic fungi are also producers of antibacterial compounds and camptothecin.

MATERIALS AND METHODS

Sample collection

I. chinensis was collected at Ba Vi National Park in 2021 and the location did not need any specific permission. Two healthy trees were divided into 5 parts, including leaf, branch, stem, flower, and root for sampling. All samples were removed from the soils and put in sterile polythene bags and transferred to the Institute of Biotechnology, Vietnam Academy of Science and Technology. Plant identification was performed at the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. Endophytic fungi were isolated based on the previous protocol used previously (Vu *et al.*, 2023). In brief, plant samples were washed with running water and then deionized water 3 times to remove soils and contaminants. Samples were immersed in 75% (v/v) ethanol for 3 min, followed by treatment with NaOCl for 3 min at room temperature. They were cut into small pieces, immersed in 75% ethanol for 1 min, then rinsed with deionized water 3 times. Samples were plated on Potato

Dextrose Agar (PDA) plates containing 0.05% penicillin and 0.1% streptomycin to inhibit endophytic bacteria growth. All dishes were then inoculated at 28 ± 2°C in the dark. After 20 days, fungal colonies grown on the plant tissues were picked and cultivated on the new PDA plates to obtain pure isolates. All pure fungal isolates were stored in PDA slants at 4°C as well as 15% (v/v) glycerol at -80°C for further experiments.

Morphological and molecular identification of endophytic fungi

All obtained fungi were inoculated on the PDA at 28 ± 2°C for 5 days to determine the growth, morphology of colonies, and pigment production. The shape of hyphae, spores, and conidiophores was visualized by a light microscope at 40X (Olympus, Japan).

The total genomic DNA of each isolate was extracted using an AllPrep Fungal DNA/RNA/Protein Kit (Qiagen) with the protocol mentioned by the manufacturer. A pair of primers, ITS1 and ITS4, were used to amplify the fungal internal transcribed spacer (ITS) DNA as previously described (Le *et al.*, 2024). The PCR reaction includes 0.1 µg of genomic DNA, 0.4 µM of ITS1 and ITS4 primers, 0.2 mM dNTPs, 1 x Taq polymerase buffer, and 1 U of Taq DNA polymerase. The amplification conditions were as follows: preheating at 94°C for 3 min, 35 cycles of 94°C for 30 s, annealing at 58°C for 20 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min. In the negative control, distilled water was used to verify the absence of contamination. Agarose gel electrophoresis was utilized to observe the PCR products and the purification step was performed using the GeneJET PCR Purification Kit (Thermo Fisher). All samples were sequenced at the National Key Laboratory of Gene Technology, Institute of Biotechnology, using the ABI Prism 3500 XL sequencer (Applied Biosystems). The obtained sequences were subsequently compared with available data from the GenBank database (NCBI) using the BLASTn program and the phylogenetic tree was built using Molecular Evolutionary Genetics Analysis v.11.0 software. The ITS sequences of strains were deposited in the GenBank with accession numbers shown in Table 1.

Cytotoxic assay

Each fungal strain was grown in 200 mL of Potato Dextrose Broth (PDB) at 28°C with the agitation speed of 180 rpm. After 7 days, the culture was extracted with 100 mL dichloromethane and 100 mL methanol with a 9:1 (v:v) ratio solvent mixture at room temperature for 60 min. After that, the extract was filtered through 25 µm porous paper and then evaporated using a vacuum rotary evaporator to obtain the dried crude extract. The crude extract was dissolved in 10% (v/v) dimethyl sulfoxide (DMSO) and used for the cytotoxic experiment.

Isolation of endophytic fungi

The sulforhodamine B (SRB) assay was utilized to test cytotoxicity against the human lung cancer A549 of 14 crude extracts. The cell line was subjected to 96-well plates with a starting optical density of 10^4 cells/well at 37°C, 95% humidity, and 5% CO_2 for 24 h. The cells were treated with 20 µg/mL of fungal extract and then fixed with 20% (w/v) trichloroacetic acid (TCA). The samples were subjected to 0.4% (w/v) SRB solution for 30 min at 37°C and de-stained by 1% (v/v) acetic acid for 3 times.

Absorbance was measured at 540 nm by ELISA Plate Reader (Biotek) and the positive control was 10 µg/mL ellipticine.

Antibacterial activity

The fungal crude extracts were evaluated for antibacterial activity using the agar well-diffusion method as mentioned previously (Vu *et al.*, 2022). *E. coli* ATCC 11105, *P. aeruginosa* ATCC 9027, and *Staphylococcus aureus* ATCC 25923 were used as test organisms. About 40 µL of each extract (2 mg/mL) was added to wells made by a sterile cork borer on the LB plates. About 40 µL of erythromycin (2 mg/mL) was utilized as positive controls and all plates were incubated at 37°C for 18 h. The diameters of bacterial growth inhibition zones were measured and the experiment was carried out in triplicate.

Determination of camptothecin

Precoated aluminum plates (TLC silica gel 60 F254 Supelco) were used to load 3 µL of the crude extract (50 mg/mL). After that, the TLC plate was visualized at λ_{254} nm under a UV lamp (Cleaver). Gradient analytical HPLC (High-performance liquid chromatography) assay was performed on an Agilent 1100 instrument. About 10 µL of crude extract was added to an octadecyl silane (ODS Agilent) (5 µm; Inertsil) column. Acetonitrile : water (20:80 to 100:00 in 30 min) was used with a flow rate of 0.6 mL/min. The peak corresponding to camptothecin was compared and calculated based on the comparison with external standard calibration curves. HPLC assay of camptothecin yielded chromatograms with a retention time of 17.7 min.

RESULTS AND DISCUSSION

Isolation of endophytic fungi isolated from *I. chinensis*

From the 10 plant parts representing a total of 2 roots, 2 stems, 2 branches, 2 leaves, and 2 flowers, a total of 14 fungal isolates with different morphological characteristics were isolated (Figure 1A). Among them, 4 fungal isolates (28.6%) were recovered from leaves, followed by roots (3; 21.4%), branches (3; 21.4%), flowers (2; 14.3%), and stems (2; 14.3%) (Figure 1B). Using a preliminary morphological examination, eight genera were recorded, including *Phyllosticta, Diaporthe, Neopestalotiopsis, Botryosphaeria, Pestalotiopsis, Lasiodiplodia, Talaromyces*, and *Colletotrichum.* The distribution of endophytic fungi isolated from *I. chinensis* collected in Bac Giang province revealed that endophytic fungi were the most dominant in the stem, followed by the leaf, flower, and root (Doan *et al.*, 2024), which was different from our study. In contrast, the highest number of culturable fungi were isolated from leaves of *T. chinensis* in Ha Giang province (Vu *et al.*, 2022). It holds the truth that the distribution of endophytic fungi may mainly depend on host plants and isolation methods.

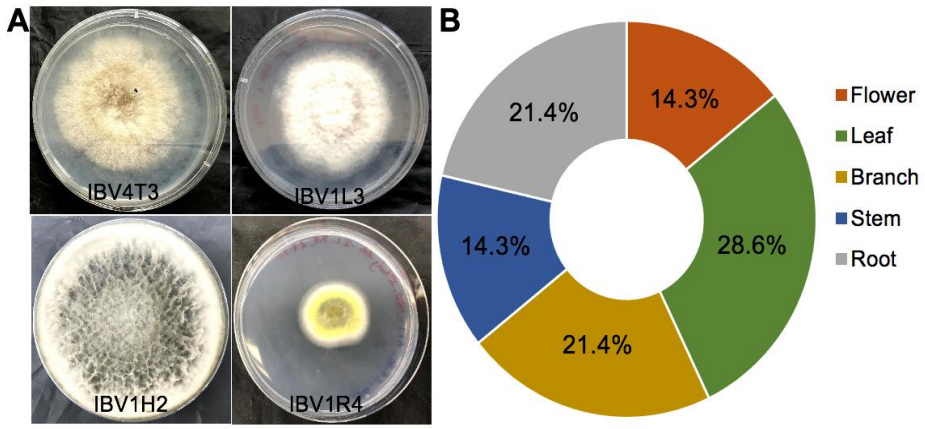

Figure 1. Colony morphology of representative fungal endophytes on the PDA plates (A) and percentage distribution of fungal endophytes associated with different plant parts (B).

Molecular identification of culturable endophytic fungi

After sequencing, 14 ITS sequences were compared to those available in GenBank to clarify the fungi at the molecular level. The BLAST analysis of 14 fungal strains revealed 98.1 – 100% identity with ITS sequences of the related species (Table 1). Similar to the morphology identification, eight genera were identified via a phylogenetic tree built from all ITS sequences (Figure 2). Based on morphological and molecular analyses, fungal isolates were identified as *Phyllosticta capitalensis* (IBV4L1), *Diaporthe passifloricola* (IBV7T4), *Diaporthe tectonigena* (IBV8T3, IBV7R2), *Neopestalotiopsis cocois* (IBV1L3), *Botryosphaeria fusispora* (IBV1H2), *Diaporthe heveicola* (IBV3T2), *Pestalotiopsis shoreae* (IBV2L2), *Colletotrichum aeschynomenes* (IBV8H1), *Lasiodiplodia lignicola* (IBV2T1), *Diaporthe tectonae* (IBV6R1), *Diaporthe howardiae* (IBV4T3), *Colletotrichum queenslandicum* (IBV3L3), and *Talaromyces rufus* (IBV1R4).

Table 1. The ITS sequence identification of the fungal isolate from *I. chinensis*

Fungal strain	High homologous strain	Sequence identity (%)	GenBank accession number
IBV4L1	*Phyllosticta capitalensis* CPC 18848^T	99.6	NR_144914
IBV7T4	*Diaporthe passifloricola* NFIF-3-21^T	99.3	NR_147595
IBV8T3	*Diaporthe tectonigena* MFLUCC 12-0767^T	99.3	NR_147589
IBV1L3	*Neopestalotiopsis cocois* MFLU 15-0220^T	99.2	NR_156312
IBV1H2	*Botryosphaeria fusispora* MFLUCC 10-0098^T	99.4	NR_121552
IBV3T2	*Diaporthe heveicola* MFLUCC 17-0329^T	98.2	NR_185666
IBV6R1	*Diaporthe tectonae* MFLUCC 12-0777^T	100	NR_147590
IBV2L2	*Pestalotiopsis shoreae* MFLU 13-0267^T	99.3	NR_154910

IBV2T1	*Lasiodiplodia lignicola* MFLUCC 11-0435^T	99.6	NR_111795
IBV8H1	*Colletotrichum aeschynomenes* ICMP 17673^T	99.6	NR_120133
IBV7R2	*Diaporthe tectonigena* MFLUCC 12-0767^T	98.1	NR_147589
IBV4T3	*Diaporthe howardiae* BRIP 59697a^T	99.1	NR_185691
IBV3L3	*Colletotrichum queenslandicum* ICMP 1778^T	99.4	NR_144796
IBV1R4	*Talaromyces rufus* CBS 141834^T	98.4	NR_170773

In our earlier work, 62 endophytic fungal strains were isolated from *I. chinensis* collected in Bac Giang province, which were classified into 11 genera, in which 4 common genera were *Diaporthe*, *Phyllosticta*, *Colletotrichum*, and *Phomopsis* (Doan *et al.*, 2024). It was consistent with our study that the *Diaporthe* genera was always the dominant genus within the endophytic fungal community present in *I. chinensis*. For *Tsuga chinensis*, *Aspergillus, Fusarium*, and *Penicillium* were found to be prevalent, which was in contrast to our current study (Vu *et al*., 2022). The variation of other genera could be due to different environmental habitats as well as host plants.

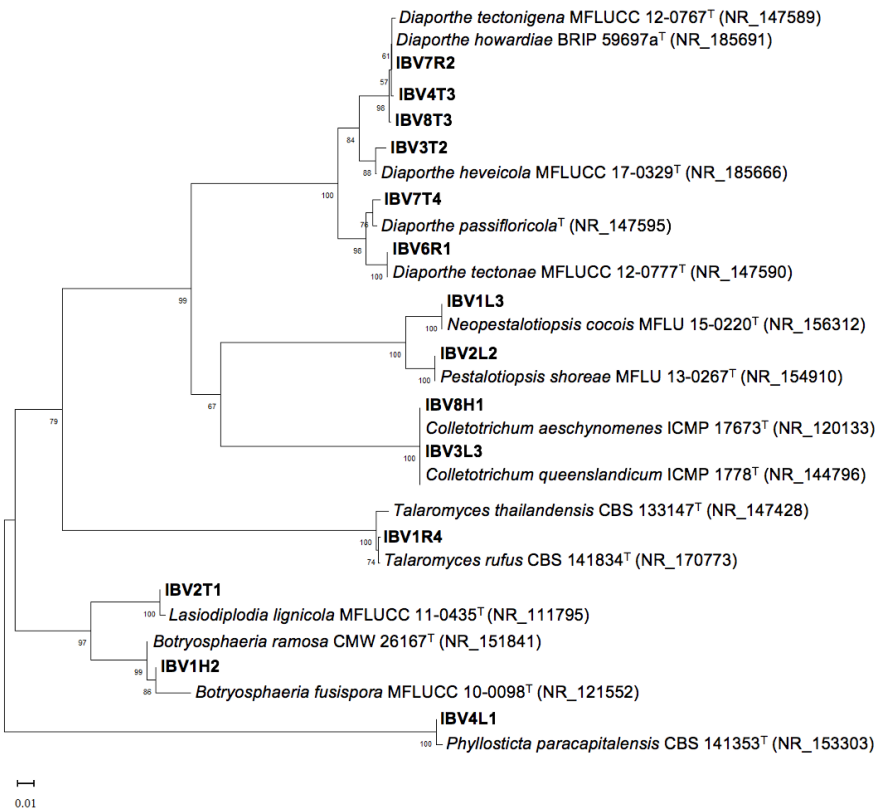

Figure 2. Neighbor-joining phylogenetic tree of 14 endophytic fungi based on ITS sequence alignments with their closest reference strains. Scale bar indicates 0.01 substitutions per nucleotide.

Anticancer activity by endophytic fungi strains

The cytotoxic activities of the crude extracts from 14 fungal strains were evaluated against the human lung adenocarcinoma A549 cell line. It turned out that percent inhibition against the A549 cell line ranged from 0.6 ± 0.3% to 71.8 ± 2.3%. *D. tectonae* IBV6R1 showed the strongest inhibitory effect on A549 cells (71.8 ± 2.3%), followed by *D. tectonigena* IBV8T3 and *D. passifloricola* IBV7T4 (Table 2). Interestingly, only *Diaporthe* spp. showed cytotoxic activity. To date, *Diaporthe* spp. are known as plant pathogens, endophytes, and saprophytes. Of note, *D. tectonae* has been reported as a phytopathogenic fungi causing leaf blight of yardlong bean (Nair *et al.*, 2021), and *D. passifloricola* was related to the stem-end rot disease in *Citrus reticulata* (Chaisiri *et al.*, 2021). The biological activities of *D. tectonae, D. passifloricola,* and *D. tectonigena* have not been explored yet to the best of our knowledge.

Table 2. Antibacterial activity and cytotoxic effect of 14 fungal endophytes

Crude extract	Percent inhibition against A549 cell line (%)	Antimicrobial activity (D-d, mm)		
		***E. coli* ATCC 11105**	***P. aeruginosa* ATCC 9027**	***S. aureus ATCC* 25923**
IBV4L1	34.7 ± 2.1	-	-	-
IBV7T4	43.6 ± 2.7	-	4.1 ± 0.6	-
IBV8T3	55.2 ± 1.4	-	-	18.7 ± 2.8
IBV1L3	19.5 ± 1.4	10.7 ± 1.1	9.8 ± 0.7	12.3 ± 2.6
IBV1H2	17.2 ± 1.8	7.4 ± 1.2	-	-
IBV3T2	0.6 ± 0.3	14.3 ± 3.4	-	1.8 ± 0.4
IBV6R1	71.8 ± 2.3	16.0 ± 2.5	16.9 ± 1.8	21.6 ± 3.1
IBV2L2	9.6 ± 0.3	4.5 ± 0.6	6.7 ± 0.6	-
IBV2T1	21.4 ± 1.1	7.0 ± 1.2	6.5 ± 0.8	11.5 ± 2.4
IBV8H1	20.1 ± 1.6	-	3.7 ± 0.8	6.4 ± 1.1
IBV7R2	4.3 ± 0.3	-	-	-
IBV4T3	9.6 ± 0.6	-	5.1 ± 1.2	-
IBV3L3	37.4 ± 0.4	3.4 ± 1.0	-	-
IBV1R4	5.3 ± 0.1	2.1 ± 0.9	1.5 ± 0.3	-
Ellipticine	96.2 ± 3.1			
Erythromycin		20.1 ± 2.4	18.4 ± 2.7	8.3 ± 1.2

Note: -, not determined.

A literature review showed that *Diaporthe* spp. are able to produce more than 106 bioactive secondary metabolites (Chaisiri *et al.*, 2021). For example,

xylariphthalide A, *cis*-4-hydroxy-6-deoxytalone, and xylarolide A produced by medicinal-plant-associated endophytic fungi showed a strong cytotoxic effect on different cancer cell lines (Hridoy *et al.*, 2022). Of note, cytotoxicity against A549 cells of crude extracts or pure compounds from *Diaporthe* spp. has not been shown yet. Since fungal genomes were estimated to contain more than 106 biosynthetic gene clusters (Quach *et al.*, 2022), it is interesting to further discover cytotoxic compounds present in the crude extract of *D. tectonae* IBV6R1.

Evaluation of the antibacterial activity of fungal extracts

All the crude extracts from the isolated fungi were separately tested for their antibacterial activity against *E. coli* ATCC 11105, *P. aeruginosa* ATCC 9027, and *S. aureus* ATCC 25923 (Table 2). The result revealed that 12 out of 14 extracts showed antibacterial activity against at least one tested bacteria, with inhibition zones ranging from 1.5 ± 0.3 to 21.6 ± 3.1 mm. Among them, only *N. cocois* IBV1L3, *D. tectonae* IBV6R1, and *L. lignicola* IBV2T1 were active against 3 pathogenic bacteria. Notably, the IBV6R1 extract displayed the best inhibition percentages against *E. coli* ATCC 11105 (16.0 ± 2.5 mm), *P. aeruginosa* ATCC 9027 (16.9 ± 1.8 mm), and *S. aureus* ATCC 25923 (21.6 ± 3.1 mm).

Given that our previous study only focused on anticancer activity (Doan *et al.*, 2024), endophytic fungi from our present study showed potent antibacterial activity. Endophytic fungus *D. caatingaensis* MT192326 from *Buchanania axillaris* was found to inhibit bacteria with the inhibition zones ranging from 15 - 22 mm, which was attributed to the presence of camptothecin (Dhakshinamoorthy *et al.*, 2021). Moreover, diaportheones, (10*S*)-diaporthin, orthosporin, phomopsolides, emodin, and phomosine produced by *Diaporthe* spp. were reported to have antibacterial activity (Xu *et al.*, 2021). These obtained results were supported by a previous study that demonstrated pathogens such as *E. coli*, *Saccharomyces cerevisiae*, methicillin-sensitive *S. aureus*, and methicillin-resistant *S. aureus* are sensitive to bioactive compounds from *D. terebinthifolii* LGMF907 (Wu *et al.*, 2022). Further studies are required to uncover the antibacterial property of *D. tectonae* IBV6R1 through extraction and structure elucidation.

Determination of camptothecin present in the IBV6R1 extract

With the strong antibacterial and anticancer activities, the extract of *D. tectonae* IBV6R1 was selected for further HPLC analysis to determine the presence of camptothecin. Firstly, the presence of camptothecin in the IBV6R1 extract was confirmed by *TLC.* It revealed that the camptothecin yielding endophytic extract gave spots corresponding to that of standard camptothecin (Figure 3A). Secondly, HPLC was utilized to detect and quantify camptothecin from the crude extract. It showed that the chromatogram of the crude extract of IBV6R1 displayed a peak with Rt at 17.75 min, matching that of authentic camptothecin (Figure 3B). The UV-visible absorption spectrum of the IBV6R1 was quite similar to that of camptothecin (Figure 3C and D). Using the standard curve, *D. tectonae* IBV6R1 was found to yield 115.6 µg/L camptothecin.

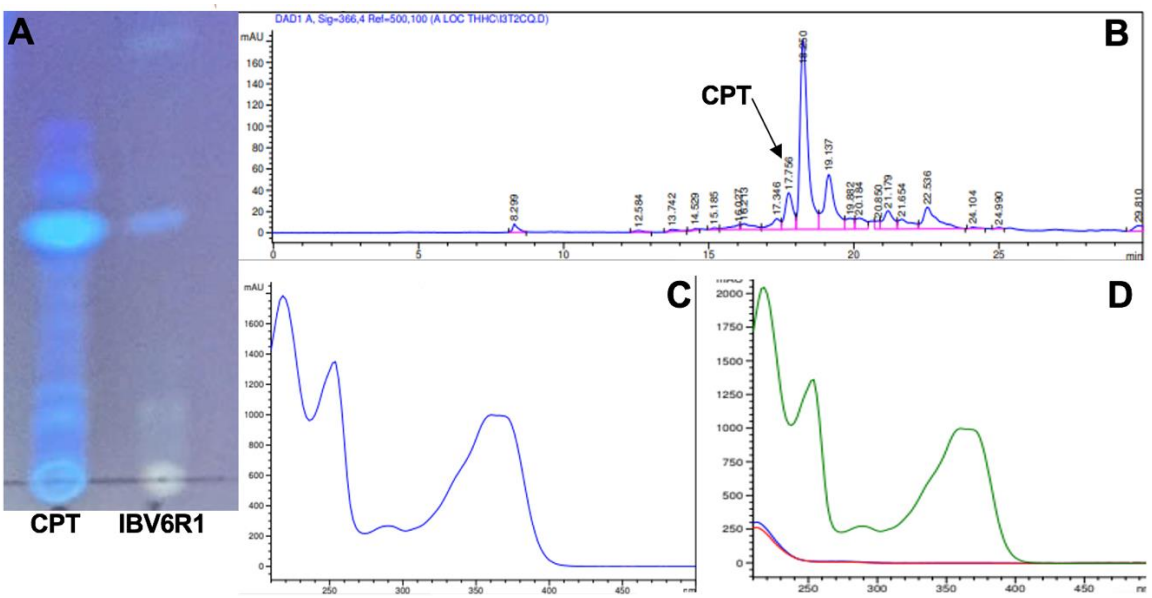

Figure 3. Identification of camptothecin produced by *D. tectonae* IBV6R1. TLC (A) and HPLC analysis (B) of the IBV6R1 extract. Comparison of UV-visible absorption spectra between authentic camptothecin (C) and the IBV6R1 extract (D).

Camptothecin is first known to be extracted from the stem bark of *Camptotheca acuminata*, which functions as an antitumor agent by inhibiting topoisomerase I interaction, resulting in cell death. It effectively treats various tumor cells, such as liver carcinoma, tumors of the head and neck, stomach and bladder cancer (Eldeghidy *et al.*, 2023). After the report of camptothecin produced by the endophytic fungus *Entrophospora infrequens*, a number of camptothecin-producing fungi have been documented (Eldeghidy *et al.*, 2023; Hridoy *et al.*, 2022). *Aspergillus terreus* OQ642314.1 was found to produce 210 µg/L camptothecin under optimized conditions (Eldeghidy *et al.*, 2023). Of note, *Phyllosticta elongata* MH458897 showed the high yield of camptothecin (747 µg/L), which was 6.5-fold higher than that of IBV6R1. At the lower level, *A. flavus* (51.7 µg/L) and *A. flavus* (37.2 µg/L) isolated from *Astragalus fruticosus* only produced 51.7 µg/L and 37.2 µg/L camptothecin, respectively (El-Sayed *et al.*, 2022). For the *Diaporthe* genera, *Diaporthe* sp. F18 and *Diaporthe caatingaensis* MT192326 were found to synthesize camptothecin (Dhakshinamoorthy *et al.*, 2021). Thus, optimization of media compositions and strain improvements are required to improve camptothecin production in the future.

CONCLUSION

The present study provides additional perspectives and insight into culturable fungi isolated from I. *chinensis* collected in Bac Giang province, and their potent antibacterial and anticancer activities. Here, 14 endophytic fungi belonging to 8 genera were isolated, among which *Diaporthe* and *Colletotrichum* were the most dominant genera. Of note, *D. tectonae* IBV6R1 was found to be a potent candidate based on strong anticancer and antibacterial activities. The fungal strain also produced 115.6 µg/L

camptothecin. This is the first report demonstrating the potential of *D. tectonae* as the prolific producer of antibacterial compounds and camptothecin. Further investigations are required to identify metabolites responsible for antibacterial activity and improve camptothecin productivity.

ACKNOWLEDGMENTS

This study was supported by the project of the Vietnam Academy of Science and Technology (VAST): "Research for the function of camptothecin composition or other substances with cancer activities in labor lever from endophytic fungi on medical plant" (Project no.: TĐCNSH.03/20-22).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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