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Distribution, cytotoxicity, and antioxidant activity of fungal endophytes isolated from *Tsuga chinensis* (Franch.) Pritz. in Ha Giang province, Vietnam

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Abstract

Purpose: An endangered *Tsuga chinensis* (Franch.) Pritz. is widely used as a natural medicinal herb in many countries, but little has been reported on its culturable endophytic fungi capable of producing secondary metabolites applied in modern medicine and pharmacy. The present study aimed to evaluate the distribution of fungal endophytes and their cytotoxic and antioxidant properties.

Methods: This study used the surface sterilization method to isolate endophytic fungi which were then identified using morphological characteristics and ITS sequence analysis. The antimicrobial and cytotoxic potentials of fungal ethyl acetate extracts were evaluated by the minimum inhibitory concentration (MIC) and sulforhodamine B (SRB) assays, respectively. Paclitaxel-producing fungi were primarily screened using PCR-based molecular markers. Additionally, biochemical assays were used to reveal the antioxidant potencies of selected strains.

Results: A total of sixteen endophytic fungi that belonged to 7 known and 1 unknown genera were isolated from *T. chinensis*. The greatest number of endophytes was found in leaves (50%), followed by stems (31.3%) and roots (18.7%). Out of 16 fungal strains, 33.3% of fungal extracts showed significant antimicrobial activities against at least 4 pathogens with inhibition zones ranging from 11.0 ± 0.4 to 25.8 ± 0.6 mm. The most prominent cytotoxicity against A549 and MCF7 cell lines (IC_{50} value < 92.4 µg/mL) was observed in *Penicillium* sp. SDF4, *Penicillium* sp. SDF5, *Aspergillus* sp. SDF8, and *Aspergillus* sp. SDF17. Out of three key genes (*dbat*, *bapt*, *ts*) involved in paclitaxel biosynthesis, strains SDF4, SDF8, and SDF17 gave one or two positive hits, holding the potential for producing the billion-dollar anticancer drug paclitaxel. Furthermore, four bioactive strains also displayed remarkable and wide-range antioxidant activity against DPPH, hydroxyl radical, and superoxide anion, which was in relation to the high content of flavonoids and polyphenols detected.

Conclusion: The present study exploited for the first time fungal endophytes from *T. chinensis* as a promising source for the discovery of new bioactive compounds or leads for the new drug candidates.

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Keywords: Antimicrobial, Antioxidant, Cytotoxicity, Endophytic fungi, Paclitaxel, *Tsuga chinensis*

Introduction

Cancer has intensively been a growing concern for humanity especially when society is at the stage of rapid development in medicine, science, and technology. As estimated by Global Cancer Observatory, there will be around 29.5 million new cancer cases, in which the implementation of cancer prevention strategies would save nearly 15 million lives (Eniu et al. 2019). Oxidative stress is known as an imbalance of reactive oxygen species (ROS) and antioxidants in the body, which can lead to cell and tissue damage interfering with cancer development (Griffiths et al. 2016; Liu et al. 2018). Indeed, ROS including both free radicals such as hydrogen peroxide and non-free oxygen radicals such as superoxide anion, singlet oxygen, and the hydroxyl radical are highly reactive molecules, which can be alleviated by antioxidants (Griffiths et al. 2016). Over the decades, several synthetic phenolic antioxidants including butylated hydroxyanisole, butylated hydroxytoluene, and propyl gallate used to treat ROS-relating diseases have been reported to cause severe side effects (Saad et al. 2007). Therefore, it is necessary to search for alternative anticancer and antioxidant agents from natural sources such as microorganisms that may overcome the complications of chemotherapy as well as prevent the risk of cancer.

To date, there is an increasing interest in endophytic fungi known as potential reservoir for bioactive compounds applying in modern medicine and pharmacy. Since plants are colonized by diverse sets of microorganisms during co-evolution, endophytic fungal communities are always varied because of host, plant distribution, ecology, and physiology (Rosa et al. 2010; Zhao et al. 2013). Of note, various reports showed that endophytic fungi isolated from medicinal plants produced not only new biomolecules, but also similar active secondary metabolites as their host (El-hawary et al. 2020). Endophytic fungi that have been exploited in many medicinal and herb plants such as *Passiflora incarnata* (L.) Medik, *Catharanthus roseus* (L.) G. Don, *Euphorbia hirta* L., and *Taxus chinensis* (Pilg.) Rehder has been considered as producers of actively plant-derived compounds that belong to different structural groups including phenols, flavonoids, alkaloids, steroids, and terpenoids (Zhou et al. 2007; Dhayanithy et al. 2019; da Silva et al. 2020; Gautam et al. 2022). Notably, the capability to produce host-specific metabolites, vincristine, vinblastine, camptothecin, podophyllotoxin, and the billion-dollar anti-cancer drug paclitaxel has been reported in fungal endophytes (Tang et al. 2020; Hridoy et al. 2022). It is believed that horizontal gene transfer likely contributes to this phenomenon during their physical contact (Richards 2011). It is evident that the PCR-based molecular markers specific for three genes 10-deacetylbaccatin III-10-O-acetly transferase (*dbat*), C-13 phenylpropanoid side chain-CoA acyltransferase (*bapt*), and taxadiene synthase (*ts*) have effectively been used to screen paclitaxel-producing fungi (Zhou et al. 2007; Kumar et al. 2019), which indicates that horizontal gene transfer takes place as result of host-fungus interactions.

Endophytic fungi that belong to the genus *Penicillium* and *Aspergillus* are viewed as producers of plant secondary metabolites like flavonoids and polyphenols that have a number of medical benefits. The ethyl acetate extract of *Penicillium oxalicum* YMG1 from *Ligusticum chuanxiong* Hort consisted of high-value polyphenols, such as hesperidin, citric acid, ferulic acid, and alternariol, which led to strong antioxidant activity against DPPH, superoxide radical and hydroxyl radical and antibacterial effects against *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 25922 (Tang et al. 2021). The endophytic fungus *Penicillium janczewskii* K.M. Zalessky was able to produce a polyphenol compound, *ρ*-hydroxybenzaldehyde that showed antibacterial activity (Schmeda-Hirschmann et al. 2005). Besides, high contents of flavonoid and polyphenol compounds in *Aspergillus flavus* L7 led to identification of rutin, gallic acid, and phlorizin as natural antioxidant agents (Patil et al. 2015). Another study identified the novel compounds seco-cytochalasins A-F, asperlactones G-H from *Aspergillus* sp. isolated from *Pinellia ternata* (Thunb) Makino, which displayed high effective cytotoxicity against human lung cancer A549 and doxorubicin-resistant human breast cancer MCF7 cell lines (Xin et al. 2019).

Tsuga chinensis is a coniferous tree species in the International Union for Conservation of Nature (IUCN) Red List, distributed only in mountains 1300–1700 m above sea level in Ha Giang province, northern Vietnam and East Asian countries (Aiello 2016). It is worth noting that *T. chinensis* is highly resistant to an aphid-like insect *Adelges tsugae* Annand that is causing widespread death of Eastern *Tsuga* species (Del Tredici and Kitajima 2004). Traditionally, the bark can also be used to treat kidney, bladder problems, diarrhea, and sores and ulcers in the mouth and throat. However, natural products with biological activities from the plant and its endophytic fungi have not been explored yet. The present study was designed to shed light on culturable endophytic fungi

from *T. chinensis* and to characterize the biological activities of fungal species. In line with these findings, we provided a new resource that yielded high levels of flavonoids and polyphenols and discovered taxol-producing endophytes. The outcomes obtained from this study open a promising avenue for conducting further in-depth investigations at the molecular and mechanistic levels.

Materials and methods

Sampling and endophytic fungi isolation

The native conifer *T. chinensis* was harvested from Dong Van (23° 15′ 30″ N 105° 17′ 24″ E), Ha Giang Province, northern Vietnam in March 2020 (Fig. S1); no specific permission was required for the location. Plant specimens were collected through guided field walks with the aid of expert plant gatherers and local ethnic minority peoples. We attempted to select three conifers distanced from each other around 3–5 m. In addition, they were roughly estimated to be 35- to 40-year-old plants whose diameter and length was around 40 cm and 13 m tall, respectively. The leaf, stem, and root samples selected from 3 trees were healthy, fresh, and free from any injury, which were subsequently transported to the laboratory of the Institute of Biotechnology, Vietnam Academy of Science and Technology. All plant specimens were sent to the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology for identification and preservation. The isolation of endophytic fungi from *T. chinensis* was performed according to a standard procedure with slight modifications (Kumar et al. 2019). In brief, the leaves, stems, and roots were cut into small segments (~ 2 × 3 cm) and washed with fresh water followed by surface sterilization via successive immersion into 70% ethanol for 30 s, 3.5% NaClO for 2 min, 70% ethanol for 2–5 s and rinsed with sterile water. The surface-disinfected samples were cut into small slices (~ 0.5 × 0.5 cm) and placed in 9-cm diameter Petri dishes (6 pieces/plate) containing Potato Dextrose Agar (PDA) supplemented with 100 mg/L streptomycin as an antibacterial agent. The plates were incubated at 28°C for 7–10 days, observed daily to check the growth of fungal colonies. For each fungal colony, single hypha was carefully sub-cultured onto fresh PDA plates to obtain pure isolates. Spores and mycelia of each fungal strain were preserved in 15% (v/v) glycerol at −80 °C. The fungal isolates producing spores were frozen at −80 °C, lyophilized under vacuum, and stored at 4°C.

Morphological, molecular identification, and isolation frequency of the endophytic fungi

Fungal isolates were cultivated on the PDA medium to observe the growth rate of hyphae, the morphology of colonies, and pigment production as described previously (Samson et al. 2011; Ngo et al. 2021). The structure of hyphae, conidia, conidiophores, and their arrangement were microscopically observed under a light microscope at 40X (Olympus, Japan). Genomic DNA of each fungal strain was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The fungal internal transcribed spacer (ITS) DNA, located between a small subunit of rRNA and a large subunit of the rRNA gene, was amplified using ITS1 (5′–TCCGTAGGTGAACCTGCGG–3′) and ITS4 (5′–TCCTCCGCTTATTGATATGC–3′) (Ngo et al. 2021). The reaction was carried out in a 25-µL final volume consisting of 0.1 µg of genomic DNA, 0.4 µM of forward and reverse primers, 0.2 mM dNTPs, 1 × Taq polymerase buffer, and 1 U of Taq DNA polymerase. For negative control, distilled water was used to verify the absence of contamination instead of genomic DNA. The amplification conditions were as follows: an initial cycle at 95 °C for 10 min, followed by 35 cycles of denaturation (95°C for 30 s), annealing (65°C for 1 min), extension (72°C for 90 s), and the process ended with a final extension at 72°C for 10 min. The PCR product was visualized on 1% (w/v) agarose gel by electrophoresis followed by purification and Sanger sequencing by First BASE Laboratories Sdn. Bhd. (Malaysia). The resulting ITS sequences were manually trimmed and edited to obtain complete sequences, which were subsequently compared with available data from GenBank databases (NCBI) using the BLASTn program. After alignment using ClustalW software v.1.81, the phylogenetic tree was computed by using the neighbor-joining method with 1000 bootstrap in Molecular Evolutionary Genetics Analysis v.7.0 (MEGA7) (Kumar et al. 2016). The ITS sequences of strains were deposited onto the GenBank (NCBI) under accession numbers as shown in Table 1. On the other hand, the isolation frequency of each fungal strain recovered from *T. chinensis* was calculated as the total number of segments yielding a given fungus divided by a total number of segments observed, expressed as a percentage (Dhayanithy et al. 2019).

Preparation of fungal extracts from fermentation broth

Each fungal strain was cultivated in 3 liters of potato dextrose broth (PDB) at 28°C, shaking at 150 rpm for 14 days. The culture was filtered by vacuum filtration, extracted with a 3X volume of ethyl acetate in a separatory funnel, and then evaporated to dryness using a vacuum rotary evaporator at 60°C (Li et al. 2014). The crude extract was weighed, dissolved in 10% (v/v) dimethyl sulfoxide (DMSO), and used for antimicrobial and cytotoxic experiments. To evaluate antioxidant activities, each crude extract was prepared by dissolving in 70% ethanol.

Table 1 Molecular identification and the colonization frequency of 16 endophytic fungi isolated from different tissues of *T. chinensis*

Endophyte code	Plant organ	GenBank accession number	Closest match	Percentage identity (%)	Isolation frequency (%)
SDF1	Stem	OM721771	*Aspergillus fumigatus*	98.11	6.25
SDF2	Leave	ON357969	*Hypoxylon griseobrunneum*	91.31	6.25
SDF3	Leave	OM721772	*Xylaria ellisii*	90.98	9.37
SDF4	Leave	OM721773	*Penicillium crustosum*	100.00	6.25
SDF5	Leave	OM721774	*Penicillium crustosum*	100.00	6.25
SDF6	Leave	OM721775	*Aspergillus austwickii*	99.64	6.25
SDF7	Leave	OM721776	*Neofusicoccum parvum*	99.40	3.13
SDF8	Leave	OM721777	*Aspergillus fumigatus*	99.85	6.25
SDF9	Stem	OM721778	*Fusarium foetens*	96.03	6.25
SDF10	Stem	ON357970	*Hypoxylon griseobrunneum*	91.22	3.13
SDF11	Stem	OM721779	*Fusarium foetens*	99.00	3.13
SDF13	Root	OM721780	*Neocosmospora magnoliae*	99.00	6.25
SDF14	Leave	OM721781	*Fusarium fujikuroi*	100.00	9.37
SDF15	Stem	OM721782	*Daldinia govorovae*	92.98	6.25
SDF17	Root	OM721783	*Aspergillus austwickii*	100.00	6.25
SDF19	Root	OM721784	*Aspergillus tubingensis*	99.82	9.37

Antimicrobial assay

The crude extract from fungal cultures was primarily screened for antimicrobial activity using the agar well- diffusion method with slight modification (Gonelimali et al. 2018). Nine different pathogenic bacteria, two Gram-negative (*Escherichia coli* ATCC 11105, *Pseudomonas aeruginosa* ATCC 9027), four Gram-positive bacteria (*Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 6538, methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591, *Enterococcus faecalis* ATCC 29212), and a yeast *Candida albicans* ATCC 10231 were used as test organisms. Freshly prepared fungal and bacterial suspensions of 100 µL were inoculated onto Sabouraud Dextrose and LB agar plates, respectively. After that, wells 6 mm in diameter were made using a sterile cork borer, and 20 µL of each extract (1 mg/mL) was added to respective wells. About 20 µL of erythromycin (1 mg/mL) and 20 µL of nystatin (1 mg/mL) were used as positive controls, while negative control wells were added with 20 µL of 10% (v/v) DMSO. The plates were then incubated at 30°C or 37°C (according to the best temperature for each microorganism to be tested) for up to 48 h. The diameters of microbial growth inhibition zones were measured after 24 h of incubation. The antimicrobial activity was determined by measuring the zone of inhibition (excluding the well diameters).

Cytotoxic potential of fungal endophytes

The cytotoxicity of crude extracts was assayed using the human lung cancer A549 and human breast adenocarcinoma MCF7 cell lines by using the sulforhodamine B (SRB) assay (Skehan et al. 1990). The A549 and MCF7 cell lines were separately seeded in 96-well plates at a density of around 10^4 cells/well and incubated at 37°C, 95% humidity, and 5% CO_2. After 24 h of incubation, the cells were treated with different concentrations of crude extract and left for 24 h. Then, the cells were fixed with cold 10% (w/v) trichloroacetic acid for 1 h at 4°C, stained with 0.4% (w/v) SRB solution for 30 min at room temperature, and then washed twice with 1% acetic acid. The results were recorded at the optical density of 540 nm in a microplate reader (BioTek EXL800). About 10 µg/mL ellipticine was used as a positive control, while 10% DMSO (v/v) was considered a negative control. The IC_{50} value is the concentration of the tested sample that inhibits 50% survival of cancer cells in comparison with the control sample grown in the same condition.

PCR based molecular screening for paclitaxel-producing fungi

Conserved genes encoding for 10-deacetylbaccatin III-10-O-acetly transferase (DBAT), C-13 phenylpropanoid side chain-CoA acyltransferase (BAPT), and taxadiene synthase (TS) were used as molecular markers to screen paclitaxel-*producing* fungi as described previously (Kumar et al. 2019). The specific primers ts-F (5′–ATCAGTCCGTCTGCATACGACA–3′), ts-R (5′–TAAGCCTGGCTTCCCGTGTTGT–3′), dbat-F (5′–ATGGCTGAC ACTGACCTCTCAGT–3′), dbat-R (5′–GGCCTGCTCCTAGTCCATCACAT–3′), bapt-F (5′–CCT

CTCTCCGCCATTGACAACAT–3′), and bapt-R (5′–GTCGCTGTCAGCCATGGCTT–3′) were synthesized according to previous studies (Zhou et al. 2007; Kumar et al. 2019). After PCR amplification, the products were analyzed on 2% (w/v) agarose gel and visualized with the Gel Doc EZ Imager (Bio-Rad Laboratories Inc.).

Antioxidant properties

The capability of fungal extracts to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) was measured as described previously with a slight modification (Kadaikunnan et al. 2015). About 100 µL of extract solution (0; 100; 200; 400 µg/mL) was added to 100 µL of 0.1 mM DPPH solution in ethanol. The mixture was kept for 30 min in darkness at room temperature and the absorbance was measured at 517 nm against an equal amount of DPPH. The percentage of DPPH scavenging activity was calculated using the following formula:

$$\text{Scavenging activity (\%)} = \left[1 - \frac{(\text{A}_{\text{sample}} - \text{A}_{\text{blank}})}{\text{A}_{\text{control}}}\right] \times 100$$

where A_{sample} is the absorbance of the reaction mixture; A_{blank} is the absorbance of 70% ethanol and fungal extract solution; and $A_{control}$ is the absorbance of 70% ethanol and DPPH solution.

The free hydroxyl radical scavenging capacity was evaluated according to the method described previously with a slight modification (Kadaikunnan et al. 2015). Briefly, the hydroxyl radical reaction consisted of 0.5 mL of 0.435 mM brilliant green, 1.0 mL of 0.5 mM $FeSO_4$, and 0.75 mL of 3% (v/v) H_2O_2. The reaction was initiated by adding 1.0 mL of crude extract solution with concentrations in the range of 0–400 µg/mL followed by incubation at 37°C for 30 min. The absorbance was measured at 624 nm and the inhibition percentage of hydroxyl radical scavenging activity was expressed as:

$$\text{Scavenging activity (\%)} = \frac{(\text{A}_s - \text{A}_0)}{(\text{A} - \text{A}_0)} \times 100$$

where A_s is the absorbance of the reaction mixture; A_0 is the absorbance of the control (70% ethanol) in the absence of the sample; and A is the absorbance without the sample and Fenton reaction system.

Superoxide radicals were carried out according to a previously published protocol with a slight modification (Li 2012). The reaction contained 900 µL of 0.05 M Tris–HCl (pH 8.2), 200 µL of crude extract (0; 100; 200; 400 µg/mL), and 80 µL of 2.5 mM pyrogallol. Following the incubation at room temperature for 5 min, the absorbance at 299 nm was measured. The superoxide radical scavenging activity was calculated as:

$$\text{Scavenging activity (\%)} = \frac{(\text{A}_{\text{control}} - \text{A}_{\text{sample}})}{(\text{A}_{\text{control}})} \times 100$$

where A_{sample} is the absorbance of the reaction, mixture and $A_{control}$ is the absorbance of the control (70% ethanol) in the reaction without the sample.

To evaluate the antioxidant activity through reducing power, the reaction mixture containing 300 µL of extracts (0; 100; 200; 400; 600 µg/mL), 300 µL of 0.2 M sodium phosphate buffer pH 7.3, and 1.5 mL of 1% (w/v) $K_3Fe(CN)_6$ was incubated at 50 °C for 25 min in the dark. After that, 300 µL of 12% (w/v) trichloroacetic acid was added to the reaction mixture and then centrifuged at 10,000 rpm for 5 min. About 1 mL of supernatant was mixed with 0.5 mL of 0.2% (w/v) $FeCl_3$. The absorbance of the mixture was measured at 700 nm. The reducing power was calculated as follows: reducing ability = A_{sample}−A, where A_{sample} is the absorbance of the mixture containing fungal extract and A was the absorbance containing deionized water instead of 0.2% (w/v) $FeCl_3$ (Rajoka et al. 2019). In all experiments, ascorbic acid was used as a positive control.

Total polyphenol and flavonoid contents

Total phenolic content was evaluated using the Folin-Ciocalteu colorimetric method described previously with a slight modification (da Silva et al. 2020). About 20 µL of crude extract dissolved in 70% ethanol was mixed with 100 µL of Folin-Ciocalteu reagent and then kept for 5 min at room temperature. The reaction was stopped by the addition of 80 µL of 4% (w/v) sodium carbonate. Absorbance at 765 nm was measured by using a microplate reader, and the results were expressed as gallic acid equivalents (GAE) in microgram per gram of dry extract (µg GAE/g FW). The total polyphenolic content of fungal extracts was established by the gallic acid standard curve, equation: $Y = 0.0043x - 0.0377$ ($R^2 = 0.9983$). All experiments were performed in triplicates.

The total flavonoid content of ethyl acetate extracts was determined spectrophotometrically based on $NaNO_2$-$Al(NO)_3$ colorimetry (Tang et al. 2020). The total flavonoids assay was conducted by mixing 30 µL of 70% ethanol extract, 10 µL of 5% (w/v) $NaNO_2$, 10 µL of 10% (w/v) $AlCl_3$, 60 µL of 1M NaOH, and 120 µL of distilled water. The mixture was incubated for 30 minutes at room temperature followed by the measurement at an optical density of 510 nm against a reagent blank. The amount of flavonoid content was calculated using a standard curve of quercetin, and the result was expressed as quercetin equivalents in microgram per gram (µg QE/g) of dry extract (µg QE/g FW). The total flavonoid content of fungal extracts was calculated using the quercetin standard curve, equation $Y = 0.0016x - 0.032$ ($R^2 = 0.9974$).

Results

Distribution and identification of culturable endophytic fungi from *T. chinensis*

A total of 16 fungal isolates with distinct morphology were successfully derived from different segments of *T. chinensis*. The greatest number of endophytic fungi were recovered from leaves (50%, 8 isolates) followed by stems (31.3%, 5 isolates) and roots (18.7%, 3 isolates) (Fig. S2A). All selected isolates were cultivated on the PDA medium and then identified by their morphology of colony and hyphae as well as spore structures (Fig. S3). As a result, 16 fungal isolates were assigned to 8 morphotypes that belonged to the phylum Ascomycota. Furthermore, ITS-based rDNA sequence analysis revealed that most sequences of these isolates were no less than 99% similar to the closest matches, except for SDF2 (91.3%), SDF3 (90.1), SDF10 (91.2%), and SDF15 (93.0%) (Table 1). The phylogenetic analysis showed that 14 fungal isolates were classified into 7 fungal genera including *Aspergillus*, *Daldinia*, *Fusarium*, *Neocosmospora*, *Neofusicoccum*, *Xylaria*, and *Penicillium* among which *Aspergillus* (31.2%), and *Fusarium* (18.7%) were the most common genera (Fig. S2B). The isolate SDF2 and SDF10 were clustered together with *Hypoxylon* as represented by the support rating of 76%, indicating that they belonged to different genera (Fig. 1). Combining with morphological characteristics, the isolates SDF2 and SDF10 have not been identified at the genus level.

Isolation frequency analysis revealed that culturable fungi in the *T. chinensis* comprised 3 frequent genera and 6 infrequent groups. *Aspergillus*, *Fusarium*, and *Penicillium* were the most prevalent genus with isolation frequency ranging from 12.5–34.4% (Table 1). Among infrequent groups, *Neofusicoccum* was the rarest genera, accounting for 3.1%, while unidentified strains SDF2 and SDF10 showed 6.25% and 3.13% of isolation frequency, respectively.

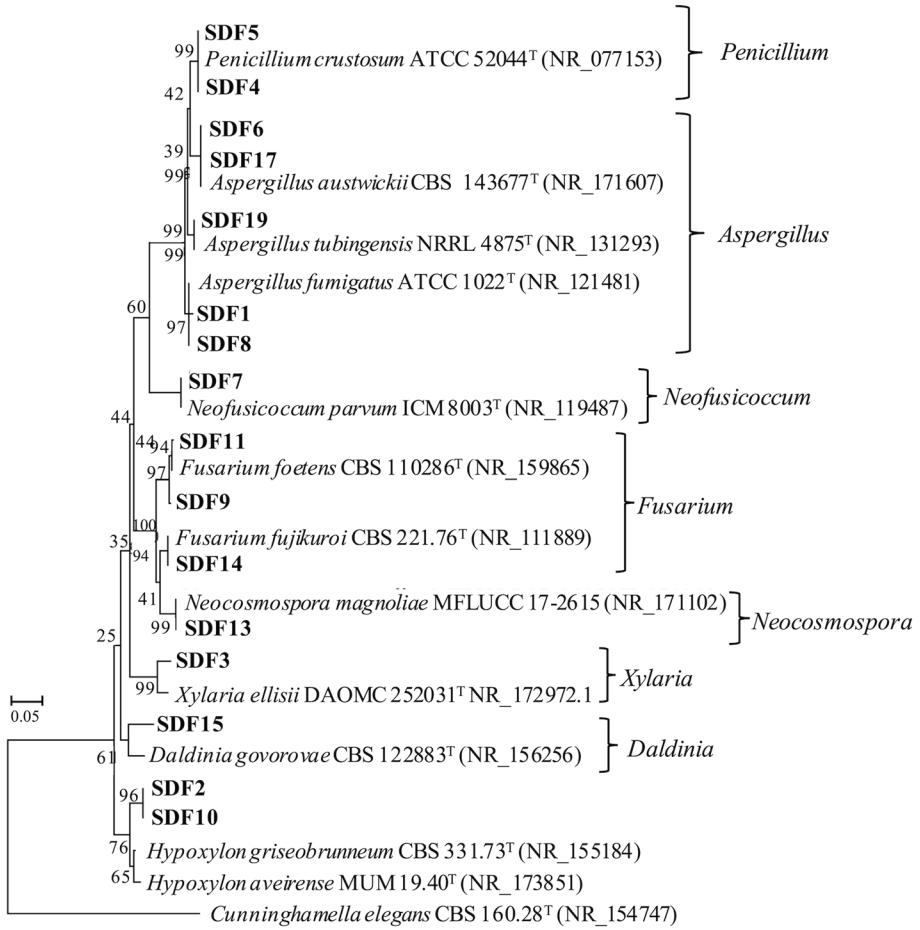

Fig. 1 Phylogenetic tree based on the ITS gene sequences of 16 the endophytic fungi isolated from *T. chinensis* showing relationship between fungal endophytes isolated from *T. chinensis* and nearest type strains. Numbers at the nodes indicate bootstrap values based on 1000 replicates and *Cunninghamella elegans* CBS 160.28^T was used as an outgroup

Antimicrobial activity by endophytic isolates

Among 16 strains, only 5 (accounting for 33.3%) endophytic fungal ethyl acetate extracts had significant inhibitory activity against a wide range of tested pathogens. These included *Aspergillus* sp. SDF1, *Penicillium* sp. SDF4, *Penicillium* sp. SDF5, *Aspergillus* sp. SDF8, and *Aspergillus* sp. SDF17 showing antimicrobial activity with inhibition zones ranging from 11.0 ± 0.4 to 25.8 ± 0.6 mm (Table 2). The growth of *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 6538, and MRSA ATCC 33591 were severely inhibited by 5 fungal extracts. The highest antibacterial activity was recorded for *Aspergillus* sp. SDF1 (25.3 ± 0.4 mm) and *Penicillium* sp. SDF4 (25.8 ± 0.6 mm) on *E. faecalis* ATCC 29212. Interestingly, only *Aspergillus* sp. SDF1 and *Penicillium* sp. SDF4 extracts were active against *C. albicans* ATCC 10231 with inhibition zones of 17.3 ± 0.4 mm and 14.2 ± 1.1 mm, respectively. Despite being classified into the same genus level and cultivated in the same condition, *Penicillium* sp. SDF4 and *Penicillium* sp. SDF5 showed a significantly different pattern of antimicrobial activity.

Anticancer activity from endophytic fungal extracts

As for anticancer activity, 4 out of 16 fungal strains showed cytotoxic activity on A549 and MCF7 cells with IC_{50} values ranging from 14.2–92.4 μg/mL, which included SDF4, SDF5, SDF8, and SDF17 (Table 2). The highest cytotoxic activity on A549 and MCF7 cells was found for SDF5 with IC_{50} values of 14.2 ± 1.5 μg/mL and 25.2 ± 1.7 μg/mL, respectively, which were around 3-fold higher than those of SDF4. On the other hand, treatment of A549 and MCF7 cells with SDF8 and SDF17 extracts led to a moderate inhibition with IC_{50} values ranging from 36.8 ± 2.8 to 50.6 ± 1.8 μg/mL. Despite the high antimicrobial activity, SDF1 showed no cytotoxic activity.

Molecular screening for paclitaxel-producing fungi

Given that paclitaxel is produced not only by endophytic fungi from *Taxus* species but also by other plants (Gangadevi and Muthumary 2009; Kumaran et al. 2009), it is interesting to evaluate the potential for paclitaxel production of *T. chinensis*-associated fungi using PCR amplification which has not been explored yet. Molecular

Table 2 Antimicrobial activity, cytotoxic effect, and the presence of paclitaxel biosynthetic genes determined in sixteen endophytic fungi isolated from *T. chinensis*

Strain	Antimicrobial activity (D-d, mm)							Cytotoxicity IC_{50} (μg/mL)		Paclitaxel biosynthetic genes		
	1	2	3	4	5	6	7	A549	MCF7	*dbat*	*bapt*	*ts*
Aspergillus sp. SDF1	22.0 ± 0.6	20.2 ± 0.4	22.6 ± 0.7	19.2 ± 1.0	15.3 ± 0.6	25.3 ± 0.4	17.3 ± 0.4	–	–	–	–	–
Unidentified SDF2	–	–	–	–	–	–	–	–	–	–	–	–
Xylaria sp. SDF3	–	–	–	–	–	–	–	–	–	+	–	–
Penicillium sp. SDF4	–	20.7 ± 0.7	20.9 ± 1.1	21.1 ± 1.0	17.7 ± 1.0	25.8 ± 0.6	14.2 ± 1.1	41.1 ± 2.7	92.4 ± 2.6	–	+	–
Penicillium sp. SDF5	21.3 ± 1.4	13.4 ± 0.8	15.5 ± 1.1	14.7 ± 0.6	11.3 ± 1.1	11.0 ± 0.4	–	14.2 ± 1.5	25.2 ± 1.7	–	–	–
Aspergillus sp. SDF6	–	–	–	–	–	–	–	–	–	–	–	–
Neofusicoccum sp. SDF7	–	–	–			–	–	–	–	–	–	–
Aspergillus sp. SDF8	19.5 ± 0.7	18.5 ± 0.3	15.3 ± 0.4	17.3 ± 1.1	15.7 ± 1.2	15.7 ± 0.4	–	36.8 ± 2.8	50.6 ± 1.8	+	–	+
Fusarium sp. SDF9	–	–	–	–	–	–	–	–	–	–	–	–
Unidentified SDF10	–	–	–	–	–	–	–	–	–	–	–	–
Fusarium sp. SDF11	–	–	–	–	–	–	–	–	–	–	–	–
Neocosmospora sp. SDF13	–	–	–	–	–	–	–	–	–	–	–	–
Fusarium sp. SDF14	–	–	–	–	–	–	–	–	–	–	–	–
Daldinia sp. SDF15	–	–	–	–	–	–	–	–	–	–	–	–
Aspergillus sp. SDF17	24.3 ± 1.1	19.4 ± 0.4	–	16.6 ± 1.0	11.3 ± 1.5	–	–	45.6 ± 5.1	45.2 ± 1.3	+	+	–
Aspergillus sp. SDF19	–	–	–	–	–	–	–	–	–	–	–	–
DMSO	–	–	–	–	–	–	–	–	–			
Erythromycin	22.1 ± 1.0	17.6 ± 1.5	29.7 ± 1.5	7.4 ± 0.6	4.3 ± 0.5	27.3 ± 1.5	–					
Nystatin	–	–	–	–	–	–	21.3 ± 1.5					
Ellipticine								0.4 ± 0.03	0.5 ± 0.05			

Microbes: (1) *Escherichia coli* ATCC 11105; (2) *Pseudomonas aeruginosa* ATCC 9027; (3) *Bacillus cereus* ATCC 11778; (4) *Staphylococcus aureus* ATCC 6538; (5) methicillin-resistant *Staphylococcus aureus* ATCC 33591; (6) *Enterococcus faecalis* ATCC 29212; (7) *Candida albicans* ATCC 10231

Result: (–) no inhibition/negative hit; (+) positive hit. Erythromycin, nystatin as well as ellipticine were used as positive control for antimicrobial and cytotoxic experiments, respectively. DMSO (10%, v/v) was employed as negative control for antimicrobial and cytotoxic experiments

detection of genes encoding for DBAT, BAPT, and TS revealed that 4 out of 16 fungi had at least one amplified gene (Table 2). Of note, the presence of *dbat* and *ts* genes were observed in SDF8, while SDF17 had *dbat* and *bapt*. Moreover, strains SDF3 and SDF4 only had positive hits of *dbat* and *bapt*, respectively. These results suggest that these strains hold a high potential for paclitaxel production, which is an interesting subject for further investigation. Based on the criteria including antimicrobial activity, cytotoxicity, and positive hits for paclitaxel biosynthetic genes, 4 strains SDF4, SDF5, SDF8, and SDF17 were selected for further studies.

Antioxidant properties

All fungal extracts displayed remarkable antioxidant activities against DPPH, hydroxyl radical, and superoxide radical, but not with reducing power (Fig. 2). Of note, only SDF8 and SDF17 extracts showed the highest antioxidant activity against DPPH radical at the concentration of 600 µg/mL, which were comparable to ascorbic acid as a positive control (96.5 ± 0.4%). The highest hydroxyl and superoxide radical scavenging activities were observed in the SDF8 (63.9 ± 0.7%) and SDF4 (69.2 ± 0.7%) extracts, respectively (Fig. 2). In contrast, the reducing power of all extracts did not increase with respect to the increasing concentrations.

Determination of the total polyphenol and flavonoid content

The total polyphenolic content of all extracts ranged from 84.3 ± 0.3 to 99.1 ± 3.2 µg GAE/g FW with strain SDF8 showing the highest polyphenol content (Fig. 3). The highest flavonoid content (111.3 ± 0.6 µg QE/g FW) was reported for the extract of strain SDF17, followed by SDF5 (99.8 ± 0.8 µg QE/g FW). In contrast, strain SDF4 produced the lowest flavonoid content (66.9 ± 1.7 µg

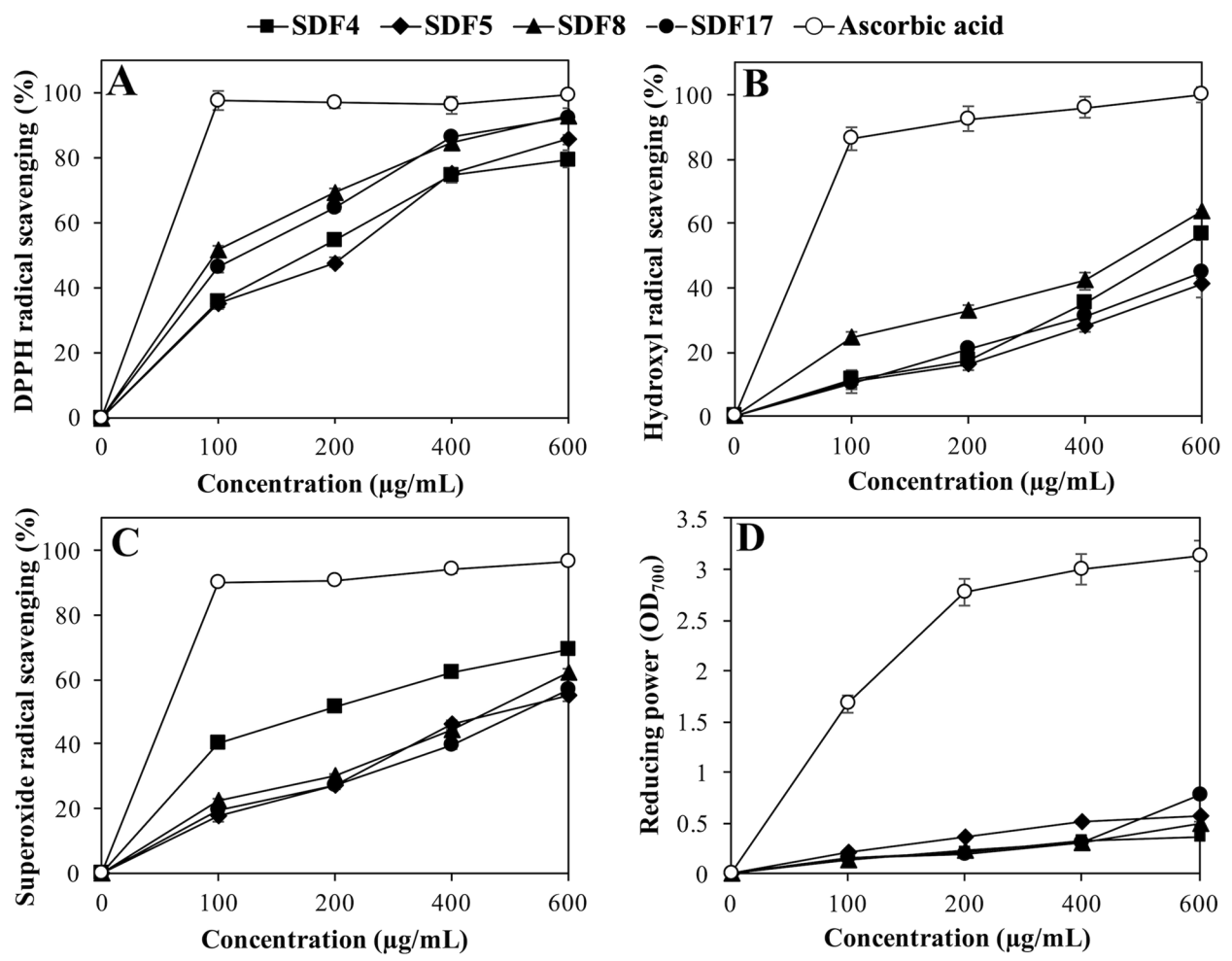

Fig. 2 Scavenging activity of ethyl acetate crude extracts from selected fungal strains on DPPH radical (**A**), hydroxyl radical (**B**), superoxide radical (**C**), and reducing power (**D**). The ascorbic acid is used as a positive control and error bars indicate the standard deviation (SD)

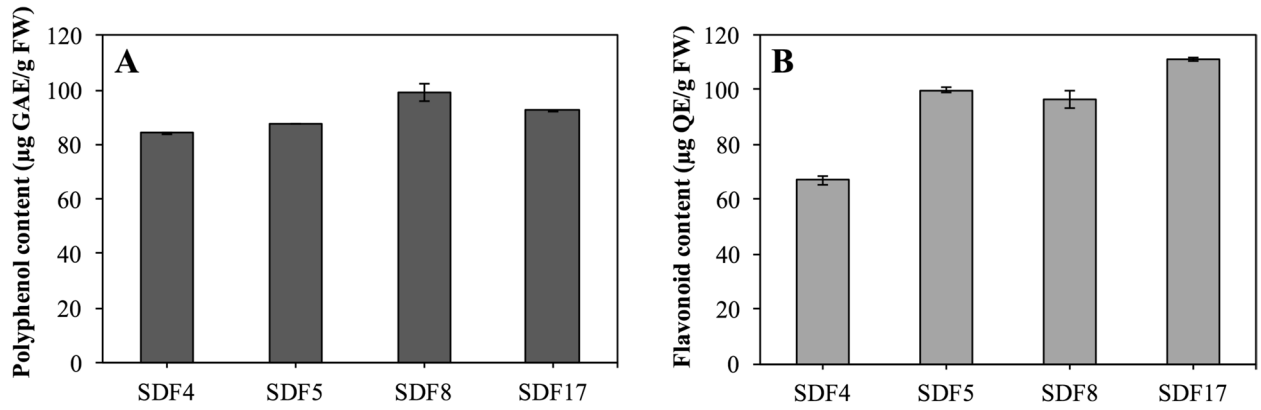

Fig. 3 Total polyphenol (**A**) and flavonoid (**B**) contents determined in the extracts of bioactive fungi

QE/g FW). It appears that all extracts comprised a relatively high level of polyphenols and flavonoids, which were relevant to the high antioxidant activities obtained.

Discussion

Endophytic fungi associated with medicinal plants are a promising source of novel bioactive compounds that hold biotechnological and medical potential. In the present study, 16 endophytic fungi were isolated from *T. chinensis*, of which 50% of the endophytes were from leaves, 31.3% from stems, and 18.7% from roots. It was nearly in agreement with the previous study showing the isolation of only 15 endophytic fungi from *Ephedra pachyclada* Boiss (Khalil et al. 2021). At the lower level, 15 fungal endophytes were recovered from the leaves and stems of five Sudanese medicinal plants (Khiralla et al. 2016). A larger number of fungi was obtained in leaves (110 isolates) and stems (3 isolates) of *Zanthoxylum simulans* Hance (Kuo et al. 2021). In line with this, previous studies proved that fungal endophytes were predominantly distributed in the leaf of herbaceous plants *Centella asiatic* (L.) Urban and *Guarea guidonia* (L.) Sleumer (Gamboa and Bayman 2001; Rakotoniriana et al. 2008). As revealed in *Catharanthus roseus*, the majority of fungal isolates originated from bark and stem (Dhayanithy et al. 2019). It is worth noting that the number and distribution of endophytic fungi vary vastly from study to study because of the many influencing factors such as isolation purpose, surface sterilization method, environment, and host-mediated factors in play (Gamboa and Bayman 2001; Rosa et al. 2010; Fernandes et al. 2015).

In this work, 14 identified strains belonged to a single division, Ascomycota, and 7 genera, namely, *Aspergillus*, *Daldinia*, *Fusarium*, *Neocosmospora*, *Neofusicoccum*, *Xylaria*, and *Penicillium*. Notably, *Aspergillus* was the most isolated genus, followed by *Fusarium*. On the other hand, in previous studies, the most common endophytes residing in plants were *Fusarium* and *Penicillium* which might assist host plants to resist abiotic and biotic stresses as well as the attack by insects or pathogens (Maciá-Vicente et al. 2008; Rosa et al. 2010; Toghueo 2019). The less diverse endophyte community observed might be due to the fact that *T. chinensis* contains various compounds actively against fungal colonization, which resulted in the dominance of the genus *Aspergillus* that were highly adapted to every ecological niche and produced a number of bioactive compounds with diverse chemical structures and biological activities (El-hawary et al. 2020). To the best of our knowledge, this is the first effort in isolating and identifying fungal endophytes isolated from *T. chinensis*.

The extracts of fungal strains isolated from *T. chinensis* also showed remarkably anti-microbial activities against a wide range of pathogens. Interestingly, all bioactive strains against pathogens were identified as *Aspergillus and Penicillium*, which confirmed the antimicrobial properties of both genera as reported previously (El-hawary et al. 2020). *Aspergillus* sp. ASCLA derived from *Callistemon subulatus* Cheel exhibited moderate to high activity against *S. aureus*, *P. aeruginosa*, and *C. albicans* (Kamel et al. 2020), while *Penicillium cataractum* SYPF 7131 from *Ginkgo biloba* L. was the most potent isolate with strong activity against five bacterial pathogens (Wu et al. 2018). The most promising strain in this study was *Penicillium* sp. SDF4 inhibiting the growth of six pathogens with inhibition zones ranging from 14.2 ± 1.1 to 25.8 ± 0.6 mm. In addition, the quantification of the total phenolic compounds and flavonoids supports the possibility that the antimicrobial effect of strain SDF4 extract is related to the presence of phenolic and flavonoid compounds that inactivate ribonucleic acid reductase blocking bacterial DNA synthesis (Rasch 2002). Hence,

Penicillium sp. SDF4 may serve as a potential source of novel antibacterial compounds.

Endophytic fungi from medicinal plants have stood out for the promising cytotoxic activity associated with anticancer properties. In the present study, *Penicillium* sp. SDF4, *Penicillium* sp. SDF5, *Aspergillus* sp. SDF8, and *Aspergillus* sp. SDF17 were found promising due to considerable inhibitory effects with IC_{50} values ranging from 14.2-92.4 μg/mL against A549 and MCF7 cells. This was consistent with various studies that proved the cytotoxic activity of the genus *Aspergillus* and *Penicillium* (El-hawary et al. 2020; Hridoy et al. 2022). In terms of the co-volution of host plants and endophytes, fungi have acquired individual genes or even gene clusters by horizontal gene transfer from host plants, which means that plant-derived compounds can be produced by fungal endophytes (Richards 2011; Hridoy et al. 2022). Positive hits for key genes *dbat* and *ts* responsible for the paclitaxel biosynthesis were detected in the *Aspergillus* sp. SDF8, while *Aspergillus* sp. SDF17 contained *dbat* and *bapt* genes. In contrast, one hit of neither *dbat* nor *bapt* was found in *Xylaria* sp. SDF3 and *Penicillium* sp. SDF4, respectively. Fungal paclitaxel has drawn particular attention from researchers in recent times because paclitaxel used in chemotherapy for many solid tumors has only been derived from rare and endangered yew trees belonging to the genus Taxus (Soliman and Raizada 2018). It is believed that both *dbat* and *bapt* are the most important genes since more than 10 enzymatic steps are taken place to synthesize paclitaxel after *ts* (Zhou et al. 2007; Kumar et al. 2019). In the genus *Aspergillus*, only *A. fumigatus* TPF-06 isolated from *Taxus* sp. was reported to have potency and sustainability to produce paclitaxel to date (Kumar et al. 2019). These results provided a shred of evidence for the presence of paclitaxel biosynthetic pathway in fungal strains SDF8 and SDF17. Using only *bapt* gene, *Guignardia mangiferae* HAA11, *Fusarium proliferatum* HBA29, and *Colletotrichum gloeosporioides* TA67 isolated from *Taxus x media* were shown to produce paclitaxel (Xiong et al. 2013), which suggested that *Xylaria sp.* SDF3 and *Penicillium* sp. SDF4 could not be excluded in quantifying paclitaxel yield. As demonstrated that paclitaxel has been also secreted by endophytes from other medicinal plants (Kumaran et al. 2009; Hridoy et al. 2022), exploiting the biosynthetic potency of paclitaxel from these promising strains SDF3, SDF4, SDF8, and SDF17 at phenotypic and genomic levels will be an interesting subject for future investigations.

Moreover, fungal endophytes also are recognized as good sources of antioxidants as an alternative to plant extracts and synthetic antioxidants. There are numerous reports demonstrating antioxidant properties of fungi related to secondary metabolites like polyphenols and flavonoids that are plant-derived compounds with a variety of significant pharmacological activities (Kumar and Pandey 2013; Shahidi and Ambigaipalan 2015; Dhayanithy et al. 2019; Rahaman et al. 2020). Here, the extracts of bioactive strains subjected to four different in vitro antioxidant experiments, DPPH, hydroxyl radical, superoxide radical, and reducing power, revealed good antioxidant activities. Similar findings have been shown in fungi recovered from some medicinal plants including *C. roseus*, *E. hirta*, *Conyza blini* H.Lév (Dhayanithy et al. 2019; Tang et al. 2020; Gautam et al. 2022). The best DPPH and superoxide radical scavenging capacities observed in fungal extracts may be attributed to their hydrogen-donating ability and superoxide dismutase-like properties, respectively indicating the potent wide-ranging antioxidant activities. *Aspergillus nidulans* ST22 and *Aspergillus oryzae* SX10 isolated from *Ginkgo biloba* L. was found to be source of phenolic and flavonoid compounds responsible for a strong antioxidant activity (Qiu et al. 2010), indicating strong correlation between total polyphenol and flavonoid contents and antioxidant activity for all the fungal extracts. The high content of polyphenol and flavonoid also provides solid evidence to support the hypothesis that phenol and flavonoid compounds are likely responsible for not only the total antioxidant capacity but also the mortality of cancer and microbial cells.

Conclusion

The present study provides new perspectives insight into culturable fungi isolated from a native conifer *T. chinensis* listed in the IUCN Red List and their potent biological activities that may stand out for promising drug candidates. Here, we isolated 16 endophytic fungi from *T. chinensis* that belonged to 7 known and one unidentified genera. Activity-based screening showed that out of 16 ethyl acetate extracts, four fungal strains *Penicillium* sp. SDF4, *Penicillium* sp. SDF5, *Aspergillus* sp. SDF8, and *Aspergillus* sp. SDF17 exhibited significant inhibitory effects against microbial pathogens, cancer cell lines, and free radicals. The biological activities obtained could be in the relation to the high content of plant-derived compounds like polyphenols and flavonoids. Surprisingly, these strains were predicted to produce paclitaxel based on the presence of key genes in the biosynthetic pathway. The study represented here compensates for the absence of research on *T. chinensis* and highlights the potential applications of endophytic fungi in the development of drugs but is required to be further investigated at the molecular and mechanistic level.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13213-022-01693-5.

Additional file 1: Fig. S1. Pictures of the whole tree (A), branch (B, C), and leaves (D) of *T. chinensis* (Franch.) Pritz collected at Ha Giang province, northern Vietnam. **Fig. S2.** Distribution of fungal strains affiliated with different plant organs (A) and genera (B) retrieved from *T. chinensis* (Franch.) Pritz. **Fig. S3.** The colony morphology of 16 endophytic fungi on PDA at 28°C isolated from *T. chinensis* (Franch.) Pritz.

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Authors' contributions
THNV and NSP conceived of this study. PCL, QAP, VTN, TTD, and NTQ designed and performed the experiments. NSP and THNV supervised and implemented the statistical analysis. THNV, NTQ, and NSP wrote the manuscript. QTP and HHC improved the writing of the manuscript. The authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
The participant has consented to the submission of this article to the journal. We confirm that the manuscript, or part of it, has neither been published nor is currently under consideration for publication. This work and the manuscript were approved by all co-authors.

Competing interests
The authors declare that they have no competing interests.

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