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Original Article

Distribution and diversity of endophytic fungi in *Gentiana rigescens* and cytotoxic activities

Li-li Xu [a], Xiao-feng Ling [a,b], Shu-juan Zhao [b], Ru-feng Wang [b,*], Zheng-tao Wang [b,*]

[a] *Institute of Science, Technology and Humanities, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China*
[b] *MOE Key Laboratory for Standardization of Chinese Medicines, SATCM Key Laboratory for New Resources and Quality Evaluation of Chinese Medicines, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China*

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ABSTRACT

Objective: In the present study, *Gentiana rigescens* was screened for fungi communities to clarify their diversity and community assemblage in hosts. Meanwhile, the identification and activity assays of the strains were also conducted.

Methods: By culture-dependent (endophytic fungi isolations from plant sections) and culture-independent (metagenomic library and cloning from plant sections) techniques, fungi communities were studied. The metagenomic library was generated using direct DNA isolation of whole plants, plant radixes, plant stems, plant leaves, plant flowers and soils around the plant. Meanwhile, endophytes were isolated from all parts of *G. rigescens* plants. After fermentation of the fungi isolations, all the isolates were evaluated for their cytotoxicity against four kinds of human cancer cell lines (HCT116, BEL7404, A549, MDA-MB-231).

Results: Eventually, 200 strains were isolated and 103 strains were further identified through the internal transcribed spacer (ITS, ITS1 and ITS2 regions) sequence by using the universal primers ITS5 and ITS4. A total of 59,106 fungal sequences corresponding to 374 putative operational taxonomic units (OTU) were identified by 454 pyrosequencing. Through 454 pyrosequencing, the main fungal genera were *Sebacina*, *Botrytis*, *Mycosphaerella*, *Boletus* and *Gibberella,* and the major fungal genera which were directly isolated were *Aspergillus, Fusarium, Penicillium* and *Alternaria*. Activity assays showed strains 5–26 (*Aspergillus* sp.) and 6–2 (*Fusarium avenaceum*) had the outstanding cytotoxicity to all the tested cell lines with IC_{50} values <5 μg/mL.

Conclusion: This study revealed the abundance of endogenetic fungal resources and a variety of genetic information in *G. rigescens* by high-throughput 454 sequencing technology and fungi isolation methods. Activity assays indicated that endophytes were a promising natural source of potential anticancer agents.

1. Introduction

Gentiana rigescens Franch., belonging to the genus *Gentiana* (Gentianaceae), was a well-known herb having been used as drug resources in traditional and folk medicine with the activities of hepatoprotection, anti-inflammation and anti-bacterial. The components of iridoid glycosides including gentiopicroside and swertiamain were important natural products that were found in *G. rigescens*. *G. rigescens* was mainly distributed in Yunnan, where has the richest species biodiversity in China, owing to the unique climate conditions and special geographical location. *G. rigescens* grown in Yunnan could have abundant endogenetic fungal resources, which might lay a foundation for the exploration of the diversity and function of *G. rigescens* and provide a basis for further biotechnological developments.

Endophytic fungi have been studied through culture-dependent isolation from host plants. Many bioactive metabolites were produced by endophytic strains (Chen et al., 2019; Gubiani et al., 2019; Li et al., 2019; Yu et al., 2019). Some endophytic fungi could work as enzymes for different biotransformation. Besides, it was reported that there were some significant interactions between host plants and fungi (Wu et al., 2019). Therefore, the endophytic fungi have been widely used and also attracted more and more attention because of their environment-friendly and time-saving characteristics.

Some literatures have shown that about 0.1%–1.0% of the endophytic fungi can be cultured. It has been suggested that the total diversity of endophytic fungi can be underestimated by culture-dependent techniques. Through the development of

* Corresponding authors.
E-mail addresses: wrffrw0801@shutcm.edu.cn (R.-f. Wang), ztwang@shutcm.edu.cn (Z.-t. Wang).

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next-generation sequencing (NGS) technologies (Xia et al., 2016; Xiong et al., 2015), such as 454 pyrosequencing, the culture-independent approach offers a powerful strategy to explain the composition of endophytic assemblages with high throughput and high accuracy in a short term. Recently, 454 pyrosequencing has been widely used in the investigation of microbial communities not only for bacterial but also for fungi (Ambayeram, Nagamani, & Trichur, 2015; Bal, Yun, Yeo, Kim, & Kim, 2016; Tao, Michael, & Ulrich, 2013).

In this study, we mainly focus on the establishment of a metagenomic library by 454 pyrosequencing from *G. rigescens* and the exploration of microbial diversity by metagenomic library and isolation of endophytes. The combination of two methods could provide better insight into the diversity evaluation of endophytic fungi of *G. rigescens*. It is also worth mentioning that, a large number of anti-tumor and anti-fungal compounds were isolated from endophytic fungi (Hafiza et al., 2019). Like the fungi of other plant hosts, the endophytes of *G. rigescens* may produce some new bioactive metabolites. Therefore, cytotoxic assays were used to evaluate the antitumor effects of metabolites produced by endophytes, which will lay a foundation for the future activity-oriented separation of compounds.

2. Materials and methods

2.1. Sample collection

G. rigescens plants were collected from Zhaotong (Yunnan, China) in September 2015, identified by Professor Li-hong Wu from Shanghai University of Traditional Chinese Medicine. Healthy and asymptomatic roots, stems, leaves and flowers were gathered, and plants with rhizosphere soil were collected and transferred to the laboratory on ice within 48 h. Fresh samples were frozen at −80 °C for DNA extraction, and some plants were refrigerated at 4 °C for the isolation of fungi.

2.2. 454 high-throughput pyrosequencing

2.2.1. Genome DNA extraction, PCR amplification and pyrosequencing

The total DNA of *G. rigescens* plants and rhizosphere soil were extracted using PowerSoil™ DNA Isolation Kits and PowerSoil™ soil DNA Isolation Kits (MoBio Laboratories, Solana Beach, CA, USA). For the construction of gene library, DNA was amplified by PCR using primer set ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The mixtures of 25 μL PCR contained 5 μL of Q5 reaction buffer (5×), 5 μL of Q5 High-Fidelity GC buffer (5×), 0.25 μL of Q5 High-Fidelity DNA Polymerase (5 U/μL), 2 μL (2.5 mM) of dNTPs, 1 μL (10 μg/ml) of each forward and reverse primer, 2 μL of DNA template, and 8.75 μL of ddH_2O. The PCR protocol was as follows: 98 °C for 2 min, followed by 25 cycles at 72 °C lasted for 30 s, and then extension at 72 °C for 30 s, with a final extension for 5 min at 72 °C. The amplicons of each sample were collected in a single tube to obtain equal molecular concentration, and an emulsion PCR was used to generate the single strands of beads required for 454 barcode pyrosequencing (Shu, He, Yue, & Wang, 2015). Using the Roche-454 GS FLX system, purified PCR amplicons were sequenced according to the protocol of Shanghai Personal Biotechnology Co., Ltd.

2.2.2. Analysis of pyrosequencing data

The sequencing data were processed by the Quantitative Insights Into Microbial Ecology (QIIME, v1.8.0) pipeline (Caporaso et al., 2010). Raw sequencing read with exact matching to the barcodes were assigned to samples and identified as valid sequences. The low-quality sequences were filtered using the following criteria (Chen & Jiang, 2014; Gill et al., 2006): sequences with length less than 150 bp, sequences with average Phred scores less than 25, sequences containing ambiguous bases, and sequences with mononucleotide repeats more than 8 bp. After chimera detection, the remaining high-quality sequences were clustered into operational taxonomic units (OTUs) by UCLUST with 97% sequence identity (Edgar, 2010). A representative sequence was selected from each OTU using default parameters. OTU taxonomic classification was based on the use of BLAST which searches for the representative sequences set against the Unite Database by the use of the best hit (Altschul et al., 997). An OTU table was further generated to record the abundance of each OTU in each sample and the taxonomy of these OTUs. In all samples, OTUs containing less than 0.001% of the total sequence were discarded.

2.2.3. Bioinformatics and statistical analysis

Sequence data analyses were performed by QIIME and R packages (v3.2.0). In the comparison of the richness and evenness of samples, OTU-level alpha diversity indices analysis including Chao1 richness estimator, Shannon diversity index and Simpson index was performed. Beta diversity analysis of the structural variation of microbial communities among different samples was carried out by using UniFrac distance metrics (Lozupone & Knight, 2005; Lozupone, Hamady, Kelley, & Knight, 2007) and the variation was visualized by principal coordinate analysis (PCoA), nonmetric multidimensional scaling (NMDS) and unweighted pair-group method with arithmetic means (UPGMA) hierarchical clustering (Ramette, 2007). The abundances of taxa at the phylum, class, order, family, genus and species levels among different samples or groups were statistically compared by Metastats (White, Nagarajan, & Pop, 2009).

2.3. Isolation and identification of endophytic fungi

Healthy and asymptomatic roots, stems, leaves and flowers of *G. rigescens* plants were thoroughly rinsed in running tap water and air-dried. Then the samples were individually surface disinfected with 75% ethanol and 5% sodium hypochlorite for 5 min and washed with sterile distilled water. Finally, the surface was dried under sterile conditions. The material was then placed in a Petri dish containing PDA medium and cultured at 28 °C. It was examined daily for the formation of mycelium or colony from the damaged surface. Then the fungus was replanted until a pure culture was obtained. Finally, a total of 200 pure culture isolates were obtained.

The fungi were identified using universal primers ITS5 and ITS4 based on internal transcribed spacer (ITS) sequence. According to the manufacturer's instructions, genomic DNA was extracted from the mycelia of strains using a Plant Genomic DNA kit (Tiangen Biotechnology Corporation). By PCR using the universal primers ITS5 and ITS4, the fungal fragments were amplified. 12.5 μL of 2× mastermix (2× Taq DNA Polymerase, 2× PCR Buffer, 2× dNTP), 1 μL of each primer (ITS5 and ITS4), 1 μL of the original template, and 9.5 μL of double distilled water constitute the PCR mixture (total volume, 25 μL). Thirty-four cycles were carried out by a denaturation step at 94 °C (60 s), an annealing step at 53 °C (45 s), and an extension step at 72 °C (90 s). Then, the last 10-min of extension step (72 °C) was performed. The reaction products were separated by an agarose gel (1.0% wt/vol), and the amplicons were purified by a gel band purification kit (Generay Biotechnology Corporation).

The phylogenetic relationships of the endophytic fungi were estimated by submitting final fungal sequences for BLAST analysis. The obtained sequences were aligned with the Clustal X2.0.1 multiple-sequence alignment software and imported into DNAMAN6.0.40. The evolutionary history was deduced by the neighbor-joining method. The bootstrap consensus tree deduced from 1000 replicates was used to represent the evolutionary history of the analytic taxa.

2.4. Cytotoxic assay

As mentioned in the literature (Xu et al., 2009; Zheng et al., 2013), the cytotoxicity of endophytic fungi against four human cancer cell lines (HCT116, BEL7404, A549, MDA-MB-231) was determined by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenylte trazoliumbromide (MTT) colorimetric assay. In briefly, the human cancer cell lines were cultured in 10% FBS DMEM medium. Then the cell lines were seeded ($4–6 \times 10^4$) in a 96-well microplate for 12 h, and each sample (10 μL) was placed in each well and incubated for 72 h at 37 °C, then was incubated with 20 μL MTT (5 mg/ml; Sigma, New York, NY, USA) for 4 h. After that, the medium was taken out and 150 μL dimethyl sulfoxide was put into each well. Finally, the plate was shaken to dissolve blue formazan crystals. Living cells were determined on a microplate reader (Labsystems, WellscanMR-2) by measuring the absorbance at a wavelength of 570 nm. The cytotoxicity of compounds to tumor cells was measured three times and was expressed by IC_{50} values.

3. Results

3.1. Microfloral diversity by metagenomics technology

Through 454 pyrosequencing, a total of 5 9106 sequencing reads were obtained from six marked samples including PR (plant roots), PS (plant stems), PL (plant leaves), PF (plant flowers), WP (whole plants) and RS (rhizosphere soils). Results showed that based on the effective 51,105 reads, the number (Operational taxonomic units) of these samples ranged from 40 to 86. Alpha diversity indices defined the richness (the Chao1 estimator and Shannon diversity index) and evenness (the Simpson index) of various taxa and pedigrees of each sample (Table 1). The beta diversity patterns between the six samples were evaluated by plotting two-dimensional Nonmetric Multidimensional Scaling (NMDS) based on unweighted UniFrac distance matrices generated by R (Fig. 1A). The clustering of all the samples was described by QIIME and R through the Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree (Fig. 1B).

Table 1
Summary of diversity indices of different samples from *G. rigescens*.

Samples	OTU numbers	Chao1	Simpson	Shannon
PR	43	44.75	0.26	1.13
PS	40	42.10	0.20	0.91
PL	86	104.75	0.40	1.81
PF	78	97.71	0.55	2.19
WP	55	61.50	0.31	1.38
RS	72	73.00	0.59	2.13

Note: PR (roots of plant), PS (stems of plant), PL (leaves of plant), PF (flowers of plant), WP (whole plants).

The taxonomic assignment of each OTU was upped to the species level through UNITE fungal ITS database supported by QIIME. The genus-level classification of the fungal ITS sequences (Fig. 2A) showed the diverse microfloral distribution in different parts of *G. rigescens*. Through 454 pyrosequencing, the main fungal genera were *Sebacina*, *Botrytis*, *Mycosphaerella*, *Boletus* and *Gibberella*. The heatmap (Fig. 2B) showed the relative proportions of the fungal lineages (50 genera) in each sample. The colors (from −2 to 2) showed the relative abundances of fungal OTUs at the 97% similarity cut-off level.

Metastats analysis was used to test differences of abundance among the six samples. From Fig. 3, the difference between generic samples was shown, and the abundance distribution of the classification units was presented, among which there were significant differences between the samples of PF and PL.

Metagenomic data was deposited into the NCBI database under the accession number SRP138972. Direct link to deposited data: https://www.ncbi.nlm.nih.gov/sra/SRP138972.

3.2. Identification of endophytic fungi

Two thousand tissue segments were screened from roots, stems, leaves and flowers of plant, which yielded 200 endophyte isolates, representing 35 morphospecies. One hundred endophyte isolates were separated from the leaf tissues, indicating that leaves were more densely colonized. The rarefaction analysis also showed that endophytic assemblages in leaves were more species-rich, because 41 species were identified from 100 isolates originated from leaves, while 31 species were identified from 50 isolates of stems, and there were 28 species identified from 40 isolates from roots. Only three species were identified among the 10 isolates from the flowers (Table 2). *Aspergillus, Alternaria, Penicillium* and *Fusarium* were the main fungal genera, while *Fusarium* sp. and *Aspergillus* sp. were the most common endophyte species, which could be isolated from every part of *G. rigescens*.

3.3. Cytotoxic activity

The ethyl acetate extracts of thirty isolates were evaluated for cytotoxic activity on human cancer cell lines. Several extracts

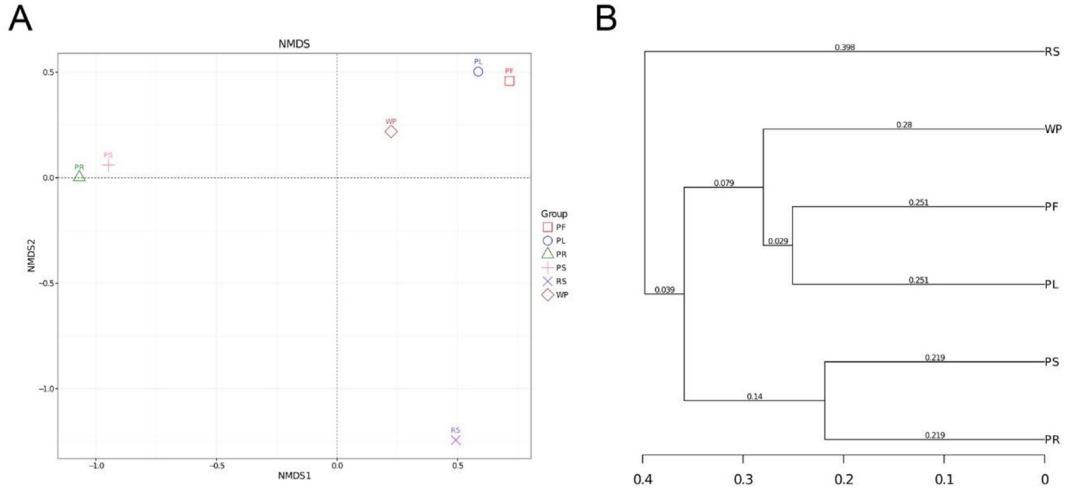

Fig. 1. Nonmetric multidimensional scaling (NMDS) (A); Unweighted pair group method with arithmetic mean (UPGMA) tree (B).

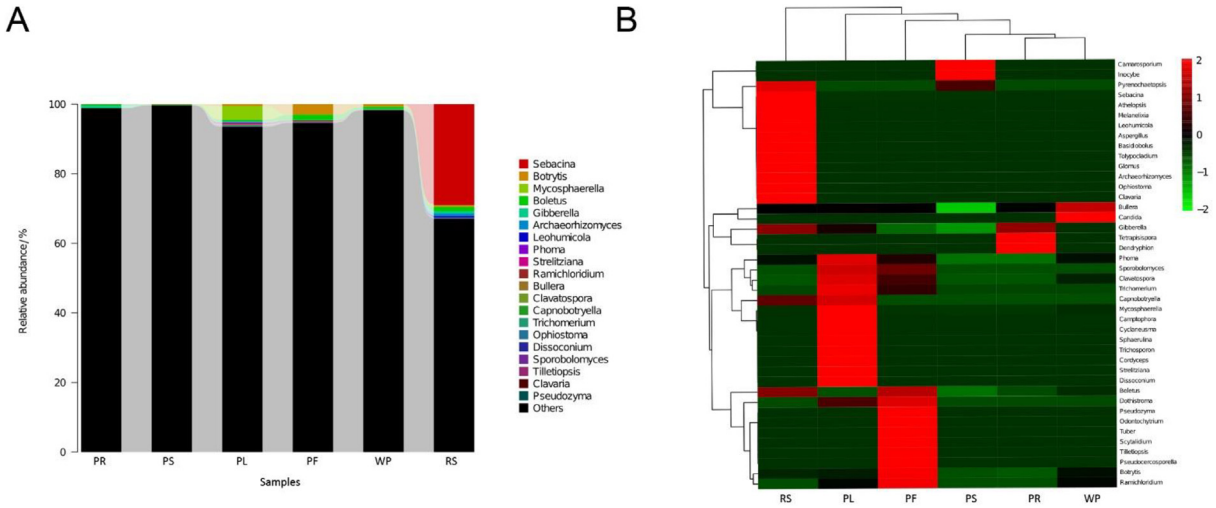

Fig. 2. Genus-level classification of the fungal ITS sequences showed the diverse microfloral distribution in different parts of *G. rigescens* (A); Heat map of genus-level classification showed the relative proportions of the fungal lineages (50 genera) in each sample (B).

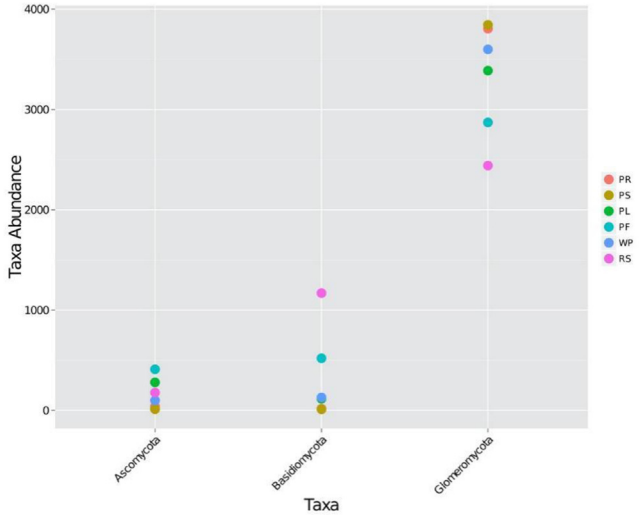

Fig. 3. Abundance distribution map between samples.

exhibited potential abilities in inhibiting four human cancer cell lines, while the others displayed no such activities. Among them, strains 5–26 and 6–2 were the most cytotoxic against tested cell lines with IC_{50} values less than 5 μg/mL, while strains 2–2, 2–5 and 2–31 also showed strong cytotoxicity with IC_{50} values ranging from 2.63 to 18.57 μg/mL (Fig. 4). In addition, 1–12, 1–23, 2–18 and 3–3 showed moderate cytotoxic activity, while other strains showed very weak or no cytotoxicity (Table S1).

4. Discussion

This project aimed to provide a profile of community microbial diversity of *G. rigescens* by using metagenomic library generation and identification after isolation for the first time. Some studies have shown that the interaction between endophytic fungi and host plants may be beneficial in some cases (Newsham, 2011). Endophytic fungi, including *Aspergillus* sp., *Alternaria* sp., *Fusarium* sp., *Penicillium* sp. and other species isolated and identified from host plants by culture-dependent analysis, have been considered to be a brand-new resource of active secondary metabolites (Chen et al., 2019; Qi et al., 2019; Tan et al., 2019), and with high-efficiency biotransformation activity of multienzyme system (Zeng et al., 2014). Some endophytic fungi were considered to promote plant growth, protect hosts from pests, and participate in the synthesis of metabolites.

Since most fungal species could not be cultured on standard growth media, the method dependent on culture inevitably underestimated the diversity of fungi. As a result of the limitations of fungi cultivation in generic laboratory conditions, studies represented only a small part of the enormous microfloral community present. With the emergence of metagenomics technology, it has become possible to thoroughly investigate the entire microbial community in plant samples and unprecedented resolutions to investigate microbial diversity were allowed. However, metagenomics analysis of *G. rigescens* has not been studied before.

To better understand the role of endophytic fungi in *G. rigescens*, it is necessary to clarify their diversity and community assemblage in hosts. Through metagenomics technology, OTU numbers, chao 1 values, simpson and shannon diversities of leaves and flowers were higher than those of roots, stems and rhizosphere soils, which indicated that the species richness, diversity and evenness of leaves and flowers were higher. At the same time, the endophytic diversity of leaves and flowers was similar, but the endophytic difference between roots and stems was small. Genus level identification of six samples showed that the most predominant genera in rhizosphere soils were *Sebacina*. *Botrytis*, *Mycosphaerella*, *Boletus* and *Gibberella* were the main genera in the whole plant.

Through isolation and identification of fungi, 35 strains were finally confirmed (Table S2; Fig. S1). There was more *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp. and *Alternaria* sp. in the samples. Meanwhile, *Aspergillus ochraceus* and *Fusarium oxysporum* were common to almost every part of plant. By comparing the culture-independent and culture-dependent methods, it was revealed that *Aspergillus* sp. and *Penicillium* sp. were common genera. Several isolates that showed strong cytotoxicity were revealed by cytotoxic activity assay. Our results showed that endophytic fungi could be effective tumor inhibitors and used for the treatment of cancer diseases.

Table 2
Endophytic fungi isolated from *G. rigescens*.

Fungi	PR	PS	PL	PF	WP
Alternaria alternata	2	2	3		7
Alternaria citri			2		2
Alternaria petroselini	2				2
Alternaria tenuissima		1			1
Aspergillus ochraceus	3	3	6		12
Aspergillus sp.			1		1
Aspergillus ustus	2	1	2	2	7
Botryosphaeria dothidea			1		1
Cladosporium allicinum	1	1	2		4
Cladosporium cladosporioides		1	2		3
Cladosporium sphaerospermum	1				1
Colletotrichum alienum	1				1
Colletotrichum gloeosporioides		1			1
Colletotrichum tropicale		1			1
Colletotrichum truncatum	1				1
Diaporthe eres			1		1
Diaporthe nobilis		1			1
Fusarium avenaceum		1			1
Fusarium equiseti	2				2
Fusarium fujikuroi		2			2
Fusarium graminearum	1	1	3		5
Fusarium oxysporum	3	3	6	1	13
Fusarium proliferatum		1	2		3
Fusarium sacchari			2		2
Fusarium solani		1			1
Fusarium venenatum	1		1		2
Fusarium verticillioides		3			3
Penicillium brasilianum	1		1		2
Penicillium chrysogenum	2	1	2		5
Penicillium decumbens	1	1	1		3
Penicillium oxalicum	2	2	1		5
Phomopsis mali	1				1
Umbelopsis dimorpha		1	1		2
Umbelopsis nana	1	1			2
Umbelopsis versiformis		1	1		2
Total No. of isolates	40	50	100	10	200

Note: PR (roots of plant), PS (stems of plant), PL (leaves of plant), PF (flowers of plant), WP (whole plants).

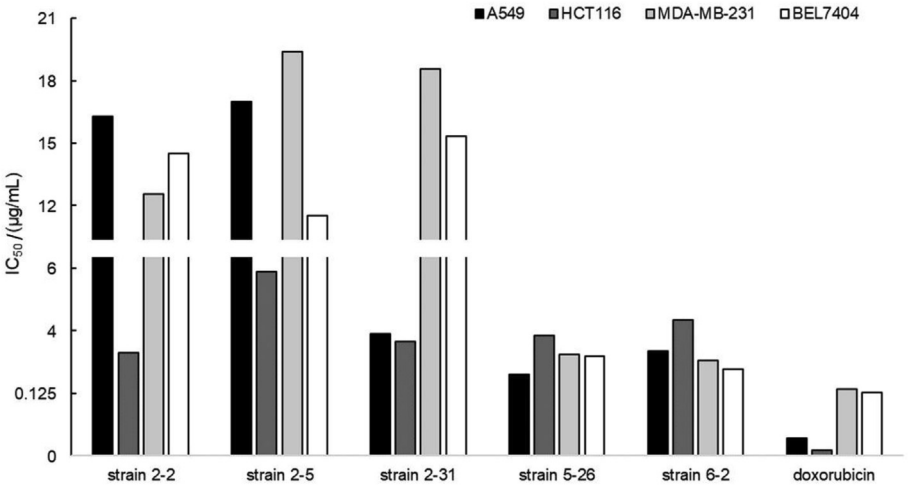

Fig. 4. *In vitro* antitumor activity of endophytic fungi.

5. Conclusion

In conclusion, our results indicated that the diversity and abundance of endophytic fungi in *G. rigescens* plants are rich. Through 454 pyrosequencing, it was found that *Botrytis*, *Boletus*, *Gibberella* and *Mycosphaerella* were the main fungal genera, while *Aspergillus, Alternaria, Penicillium* and *Fusarium* were the main directly isolated fungal genera. It was also found that endophytes from *G. rigescens* plants were potential sources of natural bioactive and brand-new components. It would be an interesting attempt to cultivate these fungi and further identify their secondary metabolites and biological function.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chmed.2020.03.007.

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