Endophytic fungi from mangrove inhibit lung cancer cell growth and angiogenesis *in vitro*

XIN LIU[1,2*], XIN WU[1*], YUEFAN MA[1,2], WENZHANG ZHANG[2], LIANG HU[2], XIAOWEI FENG[2], XIANGYONG LI[2,3] and XUDONG TANG[1-3]

[1]Guangdong Key Laboratory for Research and Development of Natural Drugs, [2]Institute of Biochemistry and Molecular Biology, and [3]Guangdong Provincial Key Laboratory of Medical Molecular Diagnostics, Guangdong Medical University, Xiashan, Zhanjiang, Guangdong 524023, P.R. China

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Abstract. The secondary metabolites of mangrove-derived endophytic fungi contain multiple substances with novel structures and biological activities. In the present study, three types of mangrove plants, namely *Kandelia candel*, *Rhizophora stylosa* and *Rhizophoraceae* from Zhanjiang region including the leaves, roots and stems were collected, and endophytic fungi were isolated, purified and identified from these mangrove plants. MTT assay was used to observe the effects of the isolated endophytic fungi on the growth of A549 and NCI-H460 lung cancer cells. The effect of the endophytic fungi on lung cancer angiogenesis *in vitro* induced by the HPV-16 E7 oncoprotein was observed. Our results showed that 28 strains of endophytic fungi were isolated, purified and identified from the three types of mangrove plants. Ten strains of endophytic fungi significantly suppressed the growth of A549 and NCI-H460 cells. The average inhibitory rates in the A549 cells were 64.4, 59.5, 81.9, 43.9, 58.3, 56.2, 48.3, 42.4, 93.0 and 49.7%, respectively. The average inhibitory rates in the NCI-H460 cells were 41.2, 49.3, 82.7, 40.7, 53.9, 52.6, 56.8, 64.3, 91.0 and 45.6%, respectively. Particularly, three strains of endophytic fungi markedly inhibited HPV-16 E7 oncoprotein-induced lung cancer angiogenesis *in vitro*. These findings contribute to the further screening of potential chemotherapeutic agents from mangrove-derived endophytic fungi.

Correspondence to: Professor Xudong Tang, Guangdong Key Laboratory for Research and Development of Natural Drugs, Guangdong Medical University, 2 Wenming Donglu, Xiashan, Zhanjiang, Guangdong 524023, P.R. China
E-mail: tangxudong2599@126.com; txd@gdmu.edu.cn

*Contributed equally

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Introduction

In 1904, the first strain of endophytic fungus was discovered. Since that date, more and more endophytic fungi have been isolated from different natural plants including mangroves (1-5). Research has demonstrated that the secondary metabolites from endophytic fungi contain multiple bioactive compounds with novel structures (1-3). The advantages of endophytic fungi include their short culture cycle, mild conditions for growth, little exposure to environmental pollution and easy industrialization. Thus, these fungi can facilitate the development of traditional pharmaceutical agents. Therefore, the identification of novel natural drugs from endophytic fungi living in different environments and ecosystems offers numerous opportunities.

Mangroves live in high saline and humid environments. Thus, numerous bioactive compounds can be generated under these environmental conditions (4,5). A large number of bioactive compounds have been isolated from endophytic fungi of mangroves, such as, flavonoids, alkaloids, terpenes, quinones, cyclic peptide compounds and fatty acids. Recently, an increased number of novel bioactive compounds have been obtained from mangrove-derived endophytic fungi, such as, novel cyclohexenone, cyclopentenone and xanthone derivatives (6), polyketides (nectriacids A-C and 12-epicitreoisocoumarinol) (7), 3-epiarigsugacin E (8), aspergifuranone and isocoumarin derivatives (9), (R)-3-demethyl purpurester A (9), aromatic butyrolactones (flavipesins A and B) (10) and sulfide diketopiperazine derivatives (penicibrocazines A-E) (11). Multiple bioactive compounds from mangrove-derived endophytic fungi have been demonstrated to exhibit inhibitory activities against acetylcholinesterase (AchE) (8), α-glucosidase (9), bacteria (10-12) and viruses (13). Particularly, accumulating evidence indicates that various bioactive compounds from mangrove-derived endophytic fungi display antitumor activities (13-18). Furthermore, studies have reported the underlying mechanisms of the antitumor effects of mangrove-derived endophytic fungi (19-24). An anthraquinone compound G503, isolated from the secondary metabolites of the mangrove endophytic fungus *Nigrospora* sp. (no. 2508), was reported to induce apoptosis in gastric cancer SGC7901 cells through the mitochondrial pathway (19). Xyloketal B, a marine compound

obtained from the mangrove fungus *Xylaria* sp. (no. 2508) from the South China Sea, was demonstrated to suppress the proliferation and migration of glioblastoma U251 cells by inhibiting the TRPM7-mediated PI3K/Akt and MEK/ERK signaling pathways (20). The marine anthraquinone derivative SZ-685C, isolated from the secondary metabolites of the mangrove endophytic fungus *Halorosellinia* sp. (no. 1403), was found to induce apoptosis in primary human non-functioning pituitary adenoma (21), breast cancer (22) and human nasopharyngeal carcinoma (NPC) cells (23), and reverse the adriamycin-resistance in breast cancer cells (24) by the inhibition of the Akt pathway. Taken together, these findings indicate that the bioactive compounds from mangrove-derived endophytic fungi can inhibit cancer cell growth via the induction of apoptosis and the inhibition of the Akt signaling pathway. However, the effect of mangrove-derived endophytic fungi on cancer angiogenesis has not yet been reported.

Angiogenesis, the development of new microvascular networks, is required for cancer invasion and metastasis and plays a key role in controlling the development and progression of a variety of cancers (25). The inhibition of cancer angiogenesis can suppress the development and progression of cancers. Therefore, the screening of angiogenic inhibitors from mangrove-derived endophytic fungi is extremely important for identifying new chemotherapeutic drugs for the prevention and treatment of cancers. There are abundant resources of mangroves in Zhanjiang. Therefore, the present study was to isolate, purify and identify endophytic fungi from Zhanjiang mangroves and explore their effects on the growth and angiogenesis of lung cancer cells.

In the present study, we isolated, purified and identified 28 strains of endophytic fungi from three types of mangrove plants, namely *Kandelia candel*, *Rhizophora stylosa* and *Rhizophoraceae*, and 10 strains of endophytic fungi significantly suppressed the growth of lung cancer cell lines, A549 and NCI-H460. Furthermore, to the best of our knowledge, we demonstrated for the first time, that three strains of endophytic fungi markedly inhibited lung cancer angiogenesis *in vitro*.

Materials and methods

Reagents. Glucose was purchased from the Tianjin Fuchen Chemical Reagents Factory (Tianjin, China). Potato dextrose agar (PDA) was obtained from Hangzhou Microbial Reagent Co., Ltd. (Hangzhou, China). Tryptone and yeast extract reagents were purchased from Oxoid Ltd. (Basingstoke, Hampshire, UK). Transfection reagent (Lipofectamine™ 2000) was obtained from Invitrogen Corp. (Carlsbad, CA, USA). *In vitro* angiogenesis assay kit (ECM625) was obtained from Millipore (Temecula, CA, USA). Fungus genomic DNA extraction kit was purchased from Bioer Technology Co., Ltd. (Tokyo, Japan). MTT kit was purchased from Beyotime Biotechnology (Shanghai, China).

Collection of mangrove plants. The healthy leaves, roots and stems of mangroves (*Kandelia candel*, *Rhizophora stylosa* and *Rhizophoraceae*) were collected from Haibin Park and Gaoqiao National Mangrove Nature Reserve (Zhanjiang, Guangdong, China). The collected leaves, roots and stems of the mangroves were washed for 2 h under running tap water and were cut into ~0.5 x 0.5 cm pieces within 72 h after collection. The surface of the fragments was sterilized by sequential immersion in 75% ethanol (C_2H_5OH) for 45 sec and 5% sodium hypochlorite (NaClO) for different times (leaves for 3 min, roots for 10 min and stems for 5 min), followed by washing four to five times with sterile distilled water.

Isolation, purification and culture of the endophytic fungi. The sterilized fragments of mangroves were dried under sterile conditions. The dried fragments were cultured in plates with PDA medium (potato extract 10.0 g/l, glucose 20.0 g/l, agar 13.0 g/l and chloramphenicol 0.1 g/l) at 28˚C, and the growth of the endophytic fungal colonies from the mangrove fragments was monitored every day. The fungal colonies which grew out from the mangrove fragments were isolated and transferred to other plates with PDA medium for purification. The purified endophytic fungal colonies were photographed.

Next, the purified endophytic fungal colonies were fermented at 28˚C in glucose peptone yeast (GPY) extract broth (tryptone 2.0 g/l, yeast extract 1.0 g/l, glucose 10.0 g/l, and sea salt 20.0 g/l) in a shaking incubator (160 rpm) at 28˚C in the dark. Seven days later, fungus culture media were filtered using nylon nets to separate the mycelia and the culture broth. Mycelia were identified by molecular analysis of the internal transcribed spacer (ITS) of the genomic DNA. The culture broths were sterilized by filtration through a 0.22-μm Millipore filter, and the filtrates were used for MTT and *in vitro* angiogenesis assays.

Molecular identification of the endophytic fungi. Genomic DNA was extracted from the separated mycelia according to the manufacturer's instructions (Bioer Technology Co., Ltd.). 18S rDNA fragments were amplified by PCR methods with universal primers. PCR primers used were: 5'-TCCGTA GGTGAACCTGCGG-3' (forward) and 5'-TCCTCCGCT TATTGATATGC-3' (reverse) (GenBank, NM_006486.2). The primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The thermocycling conditions were as follows: 94˚C for 5 min, followed by 35 cycles at 94˚C for 45 sec, 55˚C for 45 sec, and 72˚C for 60 sec, finally 72˚C for 7 min. The PCR products were detected by 1.5% agarose gel electrophoresis and DNA sequencing. The results of agarose gel electrophoresis were photographed. DNA sequences were analyzed by Sangon Biotech Co., Ltd. 18S rDNA fragment sequences of the isolated endophytic fungi were compared with those in the GenBank database using BLAST at the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA), and endophytic fungi were classified by morphologic traits and molecular identification. The phylogenetic trees were constructed by Mega 5.0 software.

Cell culture. Human lung adenocarcinoma cell line A549 and human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). Human lung cancer cell line NCI-H460 was obtained from the Chinese Academy of Sciences Cell Bank of Type Culture Collection (Shanghai, China). A549 and NCI-H460 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS). HUVEC cells were

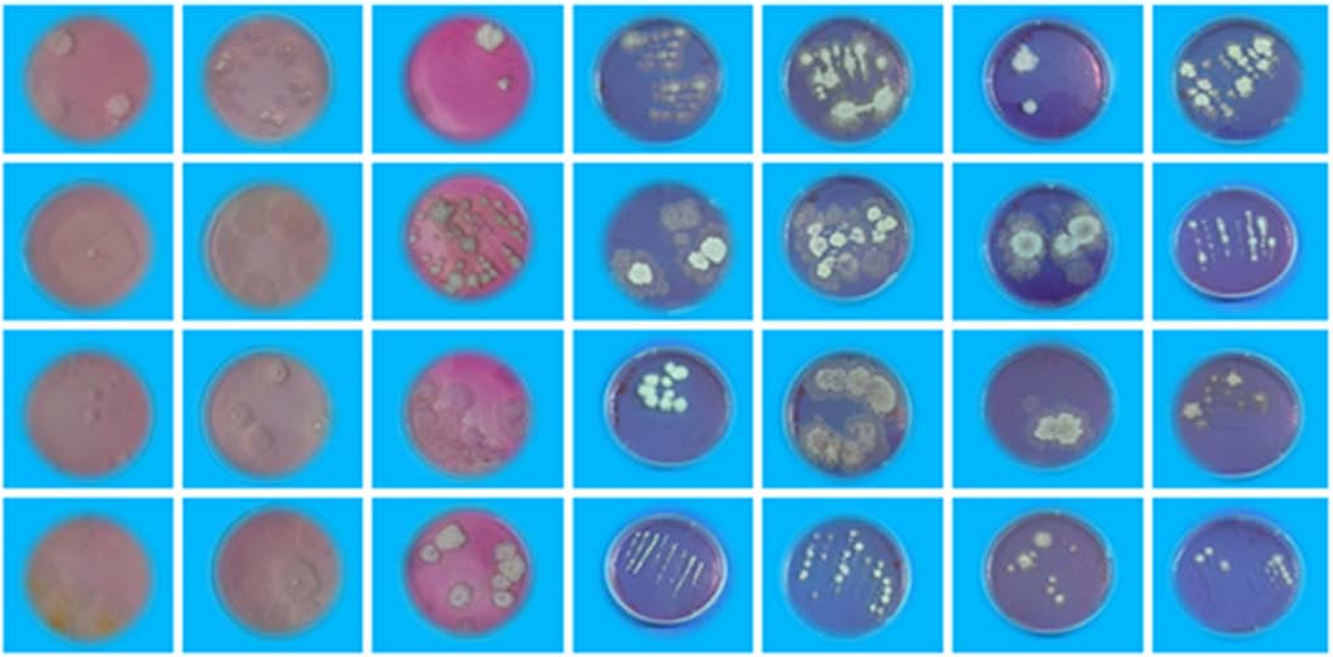

Figure 1. Twenty-eight different strains of endophytic fungi were isolated from mangroves in Zhanjiang region (magnification, x200).

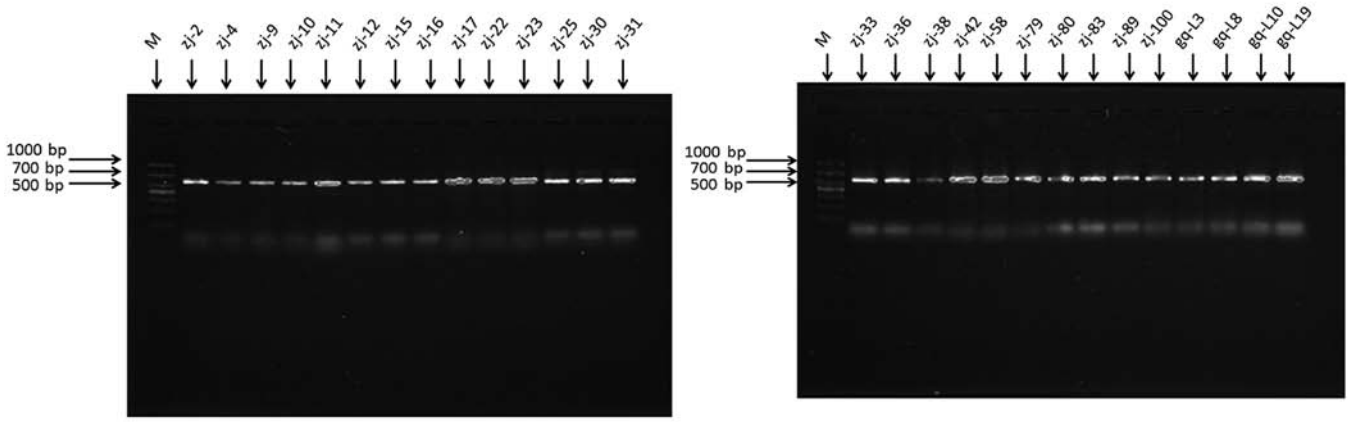

Figure 2. Agarose gel electrophoresis results of 18S rDNA of the endophytic fungi isolated from the mangroves. The 18S rDNAs extracted from 28 strains of mangrove-derived endophytic fungi were amplified by PCR. The PCR products were detected by 1.5% agarose gel electrophoresis.

grown in DEME media containing 10% FBS. All cells were maintained in a 5% CO_2 incubator at 37˚C.

Transient transfection. Transient transfection was carried out according to a previously described method (26,27). The plasmid (p-EGFP-N1-HPV-16 E7), constructed by our laboratory, was transiently transfected into A549 and NCI-H460 cells using Lipofectamine™ 2000 according to the manufacturer's instructions, wherein transfection with the empty vector (p-EGFP-N1) served as the negative controls. The cells exposed to transfection reagent alone served as mock transfection controls. The transfection efficiency was evaluated by observing green fluorescence under a fluorescence microscope, and the expression of HPV-16 E7 oncoprotein was confirmed in our previous studies (26,27).

MTT assay. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to determine the effects of endophytic fungi on the growth of A549 and H460 cells. A cell suspension was added to 96-well plates at the density of $5x10^4$/ml (100 μl/well), and cultured in a CO_2 incubator overnight at 37˚C. Afterwards, the culture medium was replaced with fresh medium, and 20 μl culture broth of the different endophytic fungi was added into the wells for culture at 37˚C for 72 h. Each culture broth of the endophytic fungi was repeated four times. Seventy-two hours later, the supernatant was removed, followed by addition of 20 μl MTT [5 mg/ml in phosphate-buffered saline (PBS)] into each well, and the cells were incubated for an additional 4 h. After removing the supernatant, 150 μl dimethyl sulfoxide (DMSO) was added into each well. The cells were incubated for

Table I. Results of the identification of isolated endophytic fungi from mangrove plants.

No.	Strain code	Similar strains	Sources of similar strains	Rates of similarity (%)	Results of identification	No.	Strain code	Similar strains	Sources of similar strains	Rates of similarity (%)	Results of identification
1	zj-2 zj-85 zj-101	*Neofusicoccum austral* (FJ037758.1)	Mangrove	100	*Neofusicoccum* sp.	15	zj-55 zj-10 zj-56 zj-88 zj-70	*Phomopsis* sp. 125AS/T (GU066687.1)	*Annona squamosa* stem	99	*Phomopsis* sp.
2	zj-16 zj-20 zj-35 zj-14 zj-67	*Neofusicoccum austral* E54ML (KF702388.1)	*Myrtus communis*	99	*Neofusicoccum* sp.	16	zj-11 zj-43	*Phomopsis* sp.139SD/S (GU066698.1)	*Spondias dulcis* seed	99	*Phomopsis* sp.
3	zj-17 zj-19 zj-77	*Neofusicoccum* sp. ZH4-E1 (FJ037734.1)	Mangrove	99	*Neofusicoccum* sp.	17	gq-L12 zj-23 zj-81	*Phomopsis* sp. ZZF08 (EU236706.10)	*Excoecaria agallocha*	99	*Phomopsis* sp.
4	zj-31	*Neofusicoccum* sp. ALG69 (KJ657714.1)	Unknown	99	*Neofusicoccum* sp.	18	gq-L17 zj-36	*Phomopsis* sp. Ac001 (JN857950.1)	*Cocos nucifera* flower	95	*Phomopsis* sp
5	zj-89 zj-93	*Neofusicoccum parvum* UY754 (EU080926.1)	Unknown	99	*Neofusicoccum* sp.	19	zj-58	*Phomopsis* sp. 20SO/L (GQ407098.1)	*Saccharum officinarum* leaf	99	*Phomopsis* sp.
6	zj-25 zj-74 zj-86	*Penicillium griseofulvu* 091402 (EU664471.1)	Mangrove	99	*Penicillium* sp.	20	zj-9 zj-37 zj-82 zj-29	*Leptosphaerulina chartarum* (GQ254687.1)	Unknown	96	*Leptosphaerulina*
7	gq-L10	*Penicillium oxalicum* NFML_CH42_88 (KM458819.1)	Unknown	99	*Penicillium* sp.	21	zj-22 zj-32	Fungal endophyte sp. AiS7(EU054418.1)	Unknown	99	Fungal endophyte sp.
8	zj-80	*Penicillium citrinum* MA-14 (HQ671192.1)	Soil	99	*Penicillium citrinum*	22	zj-38 zj-61	Fungal sp. NIS3 (KF910769.1)	*Tectona grandis* bark	91	Fungal sp.
9	zj-4 zj-45	*Pestalotiopsis* sp. 1 AE-2013 F4875 (KF746126.1)	*Bradypus variegatus*	99	*Pestalotiopsis* sp.	23	gq-L8	*Trichoderma* CHR2FC55 (KJ591703.1)	Ginger Rhizosphere soil	99	*Trichoderma*
10	zj-48 zj-100	*Pestalotiopsis* FL21 (KP689177.1)	*Huperzia serrata*	100	*Pestalotiopsis* sp.	24	zj-30 zj-98	*Hypocrea lixii* SZMC 20858 (JX173851.1)	Unknown	99	*Hypocrea lixii*

Table I. Continued.

No.	Strain code	Similar strains	Sources of similar strains	Rates of similarity (%)	Results of identification	No.	Strain code	Similar strains	Sources of similar strains	Rates of similarity (%)	Results of identification
11	gq-L1 zj-15	*Pestalotiopsis* sp. P18-11 (HQ262507.1)	Unknown	99	*Pestalotiopsis* sp.	25	zj-33	*Arthrinium* sp. 7 (HQ647335.1)	Bamboo	99	*Arthrinium* sp.
12	gq-L5 gq-L3	*Pestalotiopsis* sp. S149/2013 (KM041695.1)	Mushroom	99	*Pestalotiopsis* sp.	26	gq-L19	*Bipolaris papendorfii* CMON22 (JQ753972.1)	*Phaseolus vulgaris*	99	*Bipolaris*
13	zj-50 zj-83	*Pestalotiopsis* DBT179/2013c2 (KM041703.1)	Mushroom	99	*Pestalotiopsis*	27	zj-42 zj-68	*Fusarium oxysporum* K9 (JF807396.1)	*Pigeon pea*	99	*Fusarium oxysporum*
14	zj-39 zj-87 zj-12 zj-64	*Phomopsis* sp. 89CN/F (GU066658.1)	*Cocos nucifera* flower	99	*Phomopsis* sp.	28	zj-79	STRI:ICBG-Panama: TK1280 (KF436022.1)	*Saccharum*	99	Unknown

10 min at room temperature in order to fully dissolve the formed crystals. Absorbance values at 595 nm were measured using a microplate reader and cell viability was calculated.

In vitro angiogenesis assay. An *in vitro* angiogenesis assay kit (ECM625) was employed to analyze the formation of capillary tube-like structures according to the manufacturer's instructions. Briefly, HUVECs were seeded at a density of $5x10^3$ cells/well onto the surface of 96-well cell culture plates pre-coated with polymerized ECMatrix™. Subsequently, the conditioned media, derived from HPV-16 E7-transfected A549 or NCI-H460 cells treated with 20 μl culture broth of the different endophytic fungi, were respectively added into different wells. Tubule formation was observed under a phase-contrast microscope, and Scion image software was used to analyze the total tube length in three random view fields/well, and the average value was calculated. The experiment was repeated in triplicate.

Statistical analysis. The experiment was repeated at least three times. One way ANOVA and LSD were employed for statistical analysis using SPSS 19.0. $P<0.05$ was considered to indicate a statistically significant result.

Results

Results of the isolation, purification and identification of the endophyte fungi. Sixty-two strains of endophytic fungi were isolated from three types of mangrove plants (*Kandelia candel*, *Rhizophora stylosa* and *Rhizophoraceae*) in the Zhanjiang region. The number of endophytic fungus strains isolated from *Kandelia candel*, *Rhizophora stylosa* and *Rhizophoraceae* was 26, 20 and 16, respectively. After sequencing, 34 strains of endophytic fungi were found to have the same sequences. After removal of the repeated ones, 28 different strains of endophytic fungi were successfully isolated in the present study (Fig. 1). To further identify the 28 strains of endophytic fungi, the 18S rDNA was amplified by PCR. The results from agarose gel electrophoresis of the PCR products are shown in Fig. 2. As shown in Fig. 2, the size of the PCR products was from 500 to 750 bp, indicating that the 28 strains belonged to fungi. Compared with the sequences in the GenBank database, 28 different strains of endophytic fungi were successfully identified (Table I). Next, the phylogenetic trees were constructed using Mega 5.0 software. The phylogenetic trees of three types of mangroves, *Kandelia candel*, *Rhizophora stylosa*, and *Rhizophoraceae,* are shown in Figs. 3-5, respectively.

Results of the MTT assay. To assess the antitumor activities of the 28 isolated strains of endophytic fungi, MTT assay was performed to observe the effects of these fungi on the growth of human lung cancer cells, A549 and NCI-H460. The results from the MTT assay are shown in Table II. As shown in Table II, 10 strains of endophytic fungi, including 4 *Neofusicoccum* sp. strains (zj-2, zj14, zj-17 and zj-67), 4 *Phomopsis* sp. strains (zj-12, zj-23, zj-36 and zj-70), 1 *Leptosphaerulina* sp. strain (zj-9) and 1 *Penicillium* sp. strain (zj-25), significantly inhibited the growth of lung cancer A549 and NCI-H460 cells. The average inhibitory rates of 10 strains

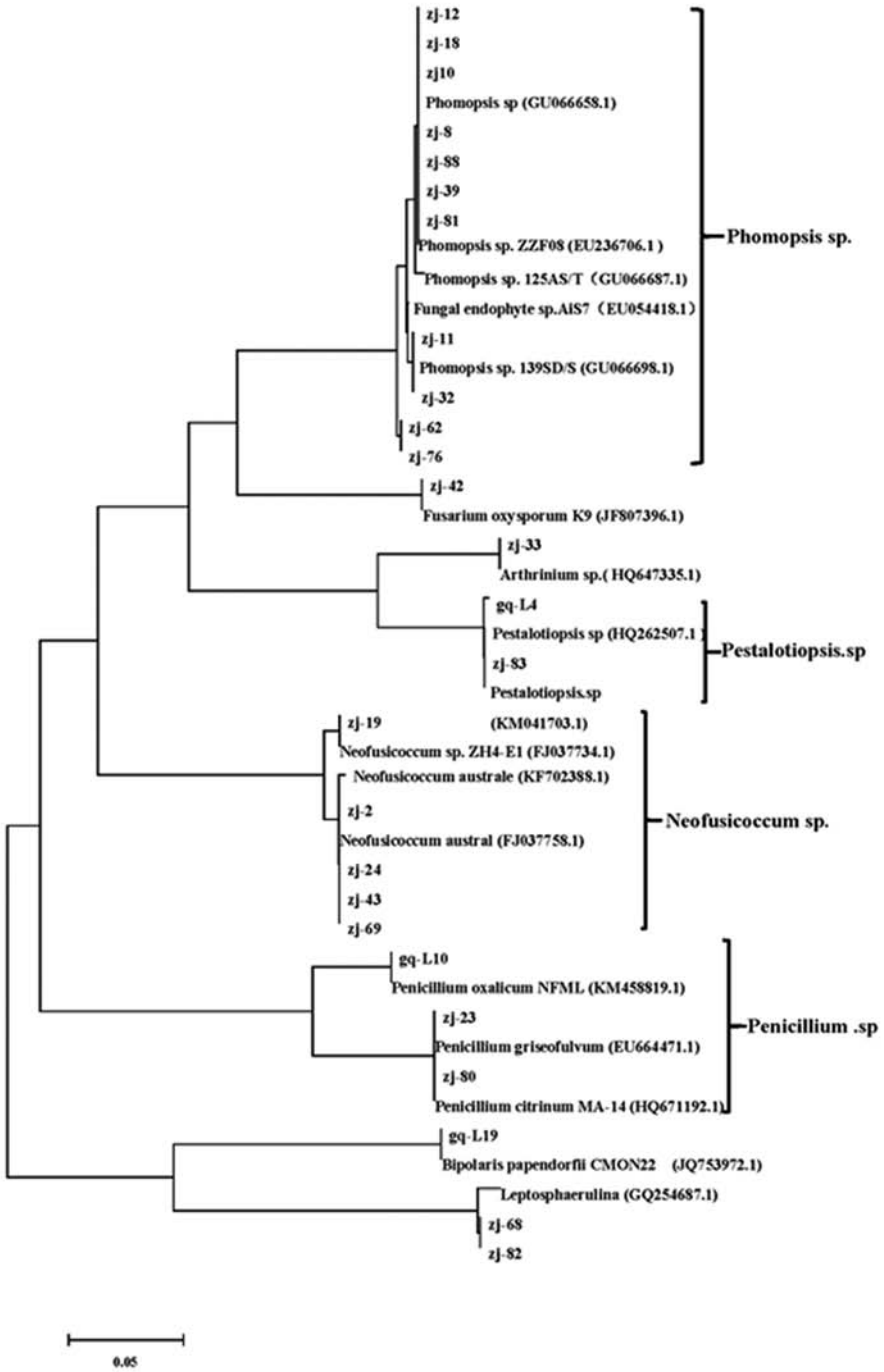

Figure 3. The phylogenetic tree of *Kandelia candel*. The sequences of endophytic fungi isolated from *Kandelia candel* were compared with those in the GenBank database using BLAST, followed by the construction of the phylogenetic tree using Mega 5.0 software.

in the A549 cells were 64.4, 59.5, 81.9, 43.9, 58.3, 56.2, 48.3, 42.4, 93.0 and 49.7%, respectively. The average inhibitory rates of 10 strains in the NCI-H460 cells were 41.2, 49.3, 82.7, 40.7, 53.9, 52.6, 56.8, 64.3%, 91.0 and 45.6%, respectively. The zj-9 and zj-17 strains showed a stronger inhibitory effects on both the A549 and NCI-H460 lung cancer cell lines. Particularly, zj-9 exhibited the strongest growth inhibitory activity in the two lung cancer cell lines.

Results of the in vitro angiogenesis assay. To further explore the underlying mechanism of the antitumor activity of the endophytic fungi, an *in vitro* angiogenesis assay was performed to observe the effects of endophytic fungi on the inhibition of lung cancer angiogenesis. In our previous studies, we found that human papillomavirus (HPV) type 16 E7 (HPV-16 E7) oncoprotein significantly enhanced lung cancer cell angiogenesis *in vitro* (26,27). Therefore, in the present study, we established an *in vitro* angiogenesis model induced by HPV-16 E7 oncoprotein. As shown in Fig. 6, HPV-16 E7 oncoprotein markedly stimulated microtubule formation (Fig. 6, image 2) as compared with the empty vector control (Fig. 6, image 1), which was

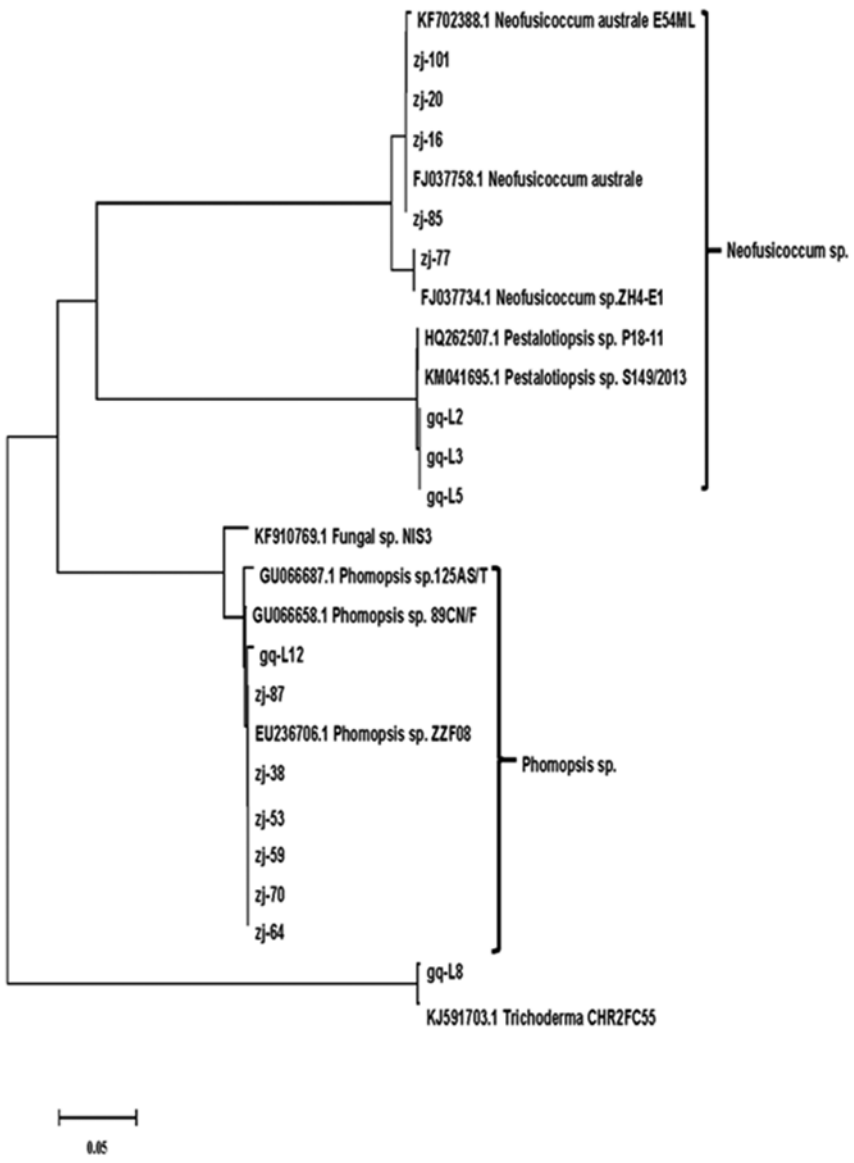

Figure 4. The phylogenetic tree of *Rhizophora stylosa*. The sequences of endophytic fungi isolated from *Rhizophora stylosa* were compared with those in the GenBank database using BLAST, followed by the construction of the phylogenetic tree using Mega 5.0 software.

in accordance with our previous studies (26,27), indicating that the model was successfully established. In the present study, we further found that endophytic fungi zj-14 (image 6), zj-17 (image 7) and zj-36 (image 10) markedly inhibited HPV-16 E7-stimulated microtubule formation (Fig. 6; P<0.01) in both A549 (Fig. 6A) and NCI-H460 (Fig. 6C) cells, which was further confirmed by total tube length (Fig. 6B and D; P<0.01). Additionally, endophytic fungus zj-23 (lane 8) inhibited HPV-16 E7-stimulated microtubule formation in the A549 cells (Fig. 6A and B; P<0.01) and endophytic fungus zj-12 (lane 5) inhibited HPV-16 E7-stimulated microtubule formation in the NCI-H460 cells (Fig. 6C and D; P<0.05).

Discussion

In the present study, we isolated 26, 20 and 16 strains of endophytic fungi from three types of mangrove plants, *Kandelia candel*, *Rhizophora stylosa* and *Rhizophoraceae*, respectively. There are a variety of endophytic fungi that may be isolated from one type of mangrove plant, but one to three endophytic fungi are dominant. We found that the dominant endophytic fungi of *Kandelia candel*, *Rhizophora stylosa* and *Rhizophoraceae* were *Pestalotiopsis* sp. (42.3%), *Pestalotiopsis* sp. (20%) and *Phomopsis* sp. (43.8%), respectively. Notably, *Neofusicoccum* sp. (31.3%) was also found to be a major advantage endophytic fungi isolated from *Rhizophoraceae* in addition to *Phomopsis* sp.

A growing body of evidence indicates that multiple bioactive compounds isolated from mangrove-derived endophytic fungi inhibit the growth of cancer cells (15-18). A new sesquiterpene named botryosphaerin F from the mangrove fungus *Aspergillus terreus* (no. GX7-3B) was reported to inhibit the growth of human breast cancer MCF-7 and leukemia HL-60 cells with IC_{50} values of 4.49 and 3.43 μM, respectively (15). Five highly oxygenated chromones, rhytidchromones A-E, were isolated from the culture broth of a mangrove-derived

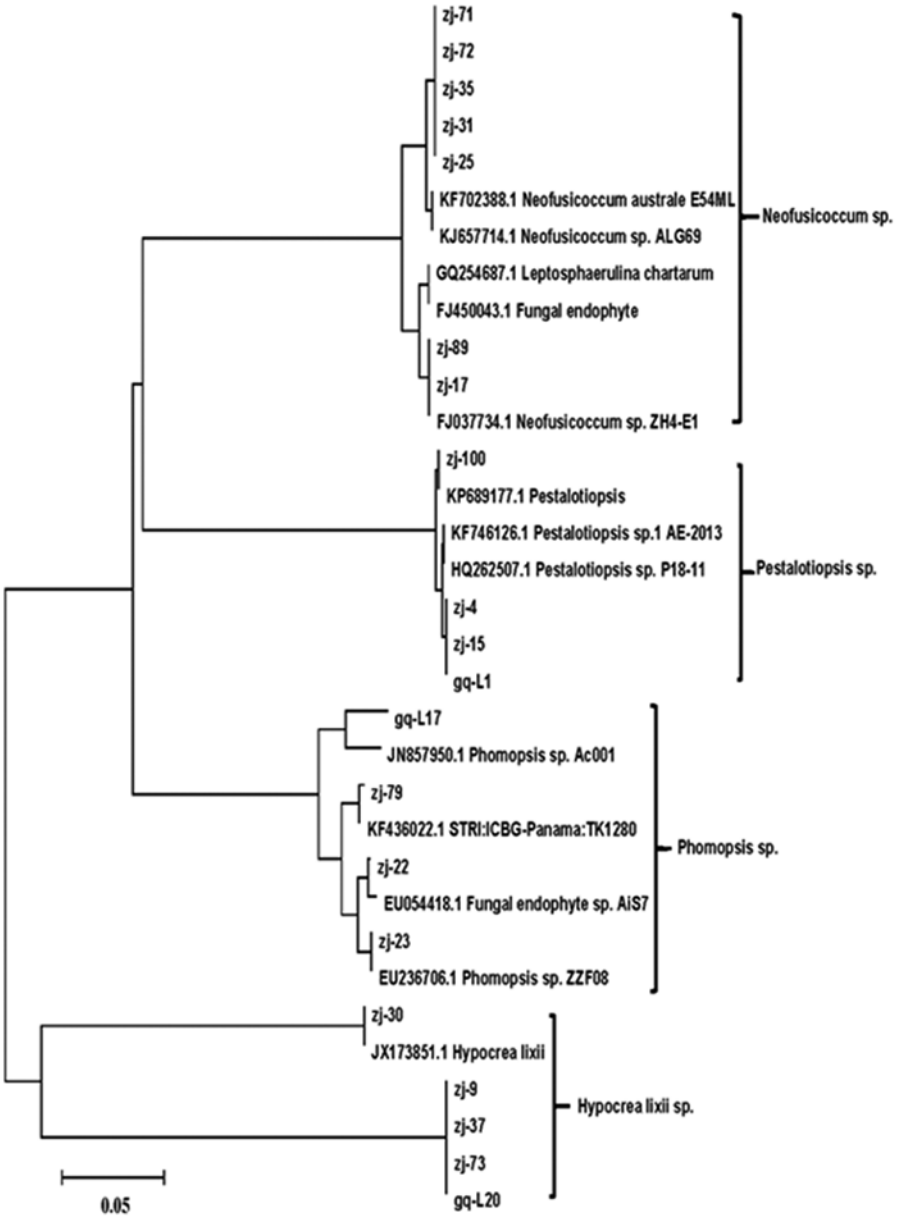

Figure 5. The phylogenetic tree of *Rhizophoraceae*. The sequences of endophytic fungi isolated from *Rhizophoraceae* were compared with those in the GenBank database using BLAST, followed by the construction of the phylogenetic tree using Mega 5.0 software.

endophytic fungus, *Rhytidhysteron rufulum*, and all compounds, except for rhytidchromone D, displayed cytotoxicity against Kato-3 cancer cells with IC_{50} values ranging from 16.0 to 23.3 μM, while rhytidchromones A and C showed activity against breast cancer MCF-7 cells with IC_{50} values of 19.3 and 17.7 μM, respectively (16). Four new lasiodiplodins (1-4) were isolated from a mangrove endophytic fungus, *Lasiodiplodia* sp. 318#., and compound 4 exhibited moderate cytotoxic activities against human cancer lines THP1, MDA-MB-435, A549, HepG2 and HCT-116 (17). Two of the three new resveratrol derivatives, namely resveratrodehydes, isolated from the mangrove endophytic fungus *Alternaria* sp. R6, were found to exhibit cytotoxic activities against human breast cancer cell line MDA-MB-435 and human colon cancer cell line HCT-116s (IC_{50} <10 μM) (18). Additionally, Tao *et al* (28) separated 87 compounds from mangrove endophytic fungus in Southern China, of which 14% of the compounds had antitumor activity. In the present study, we found that 10 strains, including *Neofusicoccum* sp. 4 strains (zj-2, zj14, zj-17 and zj-67), *Phomopsis* sp. 4 strains (zj-12, zj-23, zj-36 and zj-70), *Leptosphaerulina* sp. 1 strain (zj-9) and *Penicillium* sp. 1 strain (zj-25), significantly inhibited the growth of the lung cancer cells, A549 and NCI-H460.

Angiogenesis plays a key role in cancer invasion and metastasis. Thus, inhibition of angiogenesis is an effective strategy for cancer prevention (25,29,30). At present, multiple natural compounds have been reported in *in vitro* and *in vivo* experiments to inhibit angiogenesis, and most of them from terrestrial plants (29-33). Recently, the screening of marine medicines from the sea and its surroundings has also attracted increased attention. A number of angiogenic inhibitors from marine organisms have been found (34,35).

Table II. Results from the MTT assay.

No.	Strain code	Average inhibitory rates in A549 cells (%)	Average inhibitory rates in NCI-H460 cells (%)	No.	Strain code	Average inhibitory rates in A549 cells (%)	Average inhibitory rates in NCI-H460 cells (%)
1	zj-2 zj-85 zj-101	64.4	41.2	15	zj-55 zj-10 zj-56 zj-88 zj-70	9.4	0.14
2	zj-16 zj-20 zj-35 zj-14 zj-67	56.9	14.4	16	zj-11 zj-43	18.9	10.2
3	zj-17 zj-19 zj-77	81.9	82.7	17	gq-L12 zj-23 zj-81	16.0	37.2
4	zj-31	43.6	13.7	18	gq-L17 zj-36	48.3	56.8
5	zj-89 zj-93	20.2	39.9	19	zj-58	16.0	39.4
6	zj-25 zj-74 zj-86	49.7	45.6	20	zj-9 zj-37 zj-82 zj-29	93.0	91.0
7	gq-L10	42.4	64.3	21	zj-22 zj-32	47.7	32.1
8	zj-80	7.6	11.5	22	zj-38 zj-61	37.4	36.0
9	zj-4 zj-45	39.7	54.8	23	gq-L8	15.1	17.9
10	zj-48 zj-100	6.8	4.9	24	zj-30 zj-98	64.9	7.5
11	gq-L1 zj-15	4.6	11.7	25	zj-33	1.7	4.1
12	gq-L5 gq-L3	15.4	37.7	26	gq-L19	17.0	9.7
13	zj-50 zj-83	17.4	24.2	27	zj-42 zj-68	13.4	17.3
14	zj-39 zj-87 zj-12 zj-64	21.4	47.7	28	zj-79	11.4	16.7

Moreover, anti-angiogenic drugs can also be isolated from endophytic fungi (36-38). Altersolanol, isolated from an *Alternaria* sp. endophytic fungus, was reported to show promising anti-angiogenic activity *ex vivo*, *in vitro* and *in vivo* by the suppression of proliferation, tube formation and migration (36). The phenolic compounds, isolated from an endophytic fungus *Coccomyces proteae* collected from a Costa Rican rainforest, were reported to have anti-angiogenic

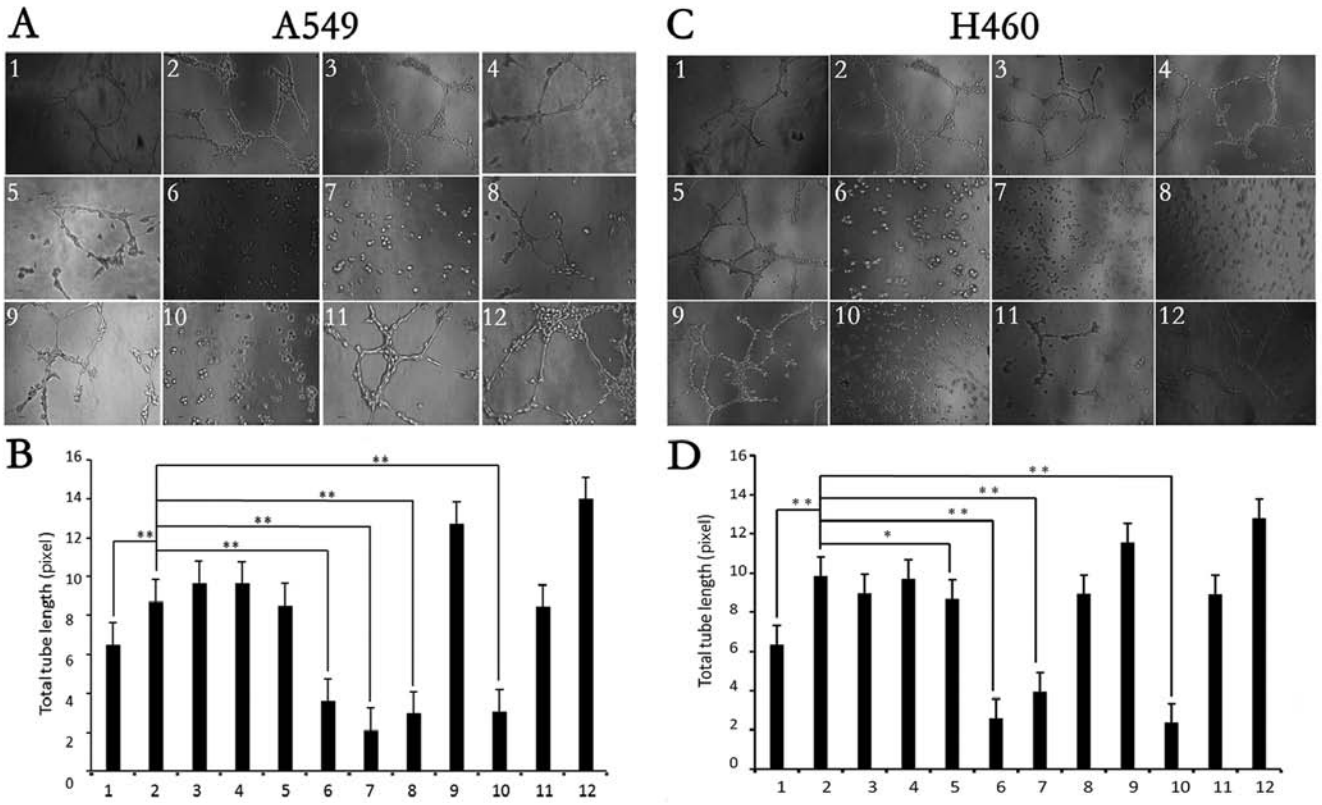

Figure 6. Effects of endophytic fungal fermentation on HPV-16 E7-stimulated lung cancer angiogenesis *in vitro*. HUVECs were incubated at 37˚C for 6-8 h in the conditioned media derived from HPV-16 E7-transfected A549 or NCI-H460 cells treated with different culture broths of endophytic fungi (zj-2, zj-9, zj-12, zj-14, zj-17, zj-23, zj-25, zj-36, zj-67 and zj-70). (A and C) Tube formation was observed under a phase-contrast microscope (magnification, x200). (B and D) The total tube length in three random view-fields/well was determined using Scion image software, and the average value was calculated. Lane 1, empty vector transfection control; lane 2, HPV-16 E7 transfection control (abbreviation, E7); lane 3, zj-2 + E7; lane 4, zj-9 + E7; lane 5, zj-12 + E7; lane 6, zj-14 + E7; lane 7, zj-17 + E7; lane 8, zj-23 + E7; lane 9, zj-25 + E7; lane 10, zj-36 + E7; lane 11, zj-67 + E7; lane 12. zj-70 + E7. All data are expressed as mean ± SD of three independent experiments; *P<0.05, **P<0.01.

activity via inhibition of capillary morphogenesis gene protein 2 (CMG2) (37). Particularly, toluquinol, isolated from marine fungus secondary metabolites, was also demonstrated to inhibit angiogenesis both *in vitro* and *in vivo* partly by the suppression of VEGF and FGF-induced Akt activation (38). In the present study, we established an HPV-16 E7 oncoprotein-induced lung cancer angiogenic model according to our previous findings (26,27), and further observed the effects of endophytic fungi which have high inhibitory effect on angiogenesis *in vitro*. We found that zj-14, zj-17 and zj-36 endophytic fungi significantly inhibited lung cancer angiogenesis *in vitro*. Notably, strain zj-9 was found to have the strongest inhibitory effect on the growth of lung cancer cells, but it did not exhibit anti-angiogenic activity, indicating that strain zj-9 may have cell cytotoxicity but not anti-angiogenic activity, and this issue warrants further study.

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