



## Antitumor and antimicrobial activities of endophytic fungi from medicinal parts of *Aquilaria sinensis*\*

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**Abstract:** The purpose of this study was to isolate and characterize endophytic fungi from the stem tissue which can produce fragrant ingredients in *Aquilaria sinensis* (also called agarwood) to determine their antitumor and antimicrobial activities. Twenty-eight fungal endophytes were isolated from agarwood by strict sterile sample preparation and were classified into 14 genera and 4 taxonomic classes (Sordariomycetes, Dothideomycetes, Saccharomycetes, and Zygomycetes) based on molecular identification. Of the 28 isolates, 13 (46.4%) showed antimicrobial activity against at least one of the test strains by the agar well diffusion method, and 23 isolates (82.1%) displayed antitumor activity against at least one of five cancer cell lines by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The diameters of inhibition zones of YNAS07, YNAS14, HNAS04, HNAS05, HNAS08, and HNAS11 were equal to or higher than 14.0 mm against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *B. subtilis*, *Aspergillus fumigatus*, and *B. subtilis*, respectively. The inhibition rates of YNAS06, YNAS08, and HNAS06 were not less than 60% to 293-T, 293-T, and SKVO3 cells, respectively. These results suggest that the endophytic fungi associated with agarwood will provide us with not only useful micro-ecological information, but also potential antimicrobial and anti-tumor agents.

**Key words:** Agar diffusion method, Agarwood, Antimicrobial bioactivity, Antitumor bioactivity, Endophytic fungi  
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### 1 Introduction

In recent years, researchers have begun to realize that plants may serve as a repository of untold numbers of organisms known as endophytes (Bacon and White, 2000; Strobel, 2002). Most endophytes are capable of synthesizing bioactive compounds that may provide plants with a defense against pathogens, and some of these compounds have proven useful for novel drug discovery (Guo et al., 2008; Yan et al., 2011). Since the chemical constituents from medicinal plants were complex, more and more endophytic fungi with novel metabolites of pharmaceutical im-

portance were isolated from medicinal plants, and a series of new and useful compounds were obtained (Huang et al., 2007; Guo et al., 2008; Kusari et al., 2009). No doubt, exploiting a variety of new natural products from endophytic fungi of medicinal plants has become a hot spot of new drug research.

Agarwood (this refer in particular to the stem tissue which can produce fragrant ingredients in *Aquilaria sinensis*) is a fragrant wood that has been widely used as traditional Chinese medicine and fragrance additive two thousand years ago (He et al., 2005). It is formed from *A. sinensis* (Lour.) Gilg. (Thymelaeaceae) which is the only origin species of agarwood in China (Liu, 1999; Qi, 1995). In recent years, many of the chemical ingredients have been isolated from agarwood (Yang and Chen, 1983; 1986; Yang et al., 1989a; 1989b; 1990; Yang, 1998; Yagura et al., 2003). By investigation of interrelated

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studies, agarwood has significant anticancer activities (Gunasekera *et al.*, 1981), analgesic and anti-inflammatory activities (Zhou *et al.*, 2008), and anti-depression activities (Okugawa *et al.*, 1993; 1996). Endophytes from agarwood may be a rich source of antimicrobial and antitumor agents with novel mechanisms of action.

As one of the renowned traditional medicines, despite great progress toward understanding phytochemical constituents and pharmacological activities of agarwood, the micro-ecology and bioactivity of endophytic fungi associated with agarwood have not been exploited. For this reason, systematic investigation of endophytic fungi associated with agarwood and evaluation of their bioactive secondary metabolites were necessary. This can provide researchers with genetic information and may allow for new natural products with higher antimicrobial and antitumor activities to be found. The present study describes the endophytic fungi isolated from agarwood growing in two different rainforest areas, and reports their antimicrobial and antitumor activities.

## 2 Materials and methods

### 2.1 Source of endophytic fungi

Samples of agarwood were collected randomly from the tropical rainforests in Yunnan and Hainan Provinces of China in March 2009 and were identified. Plant samples were tagged, stored at 4 °C in a clean plastic bag, and taken to the laboratory for isolation of endophytic fungi.

### 2.2 Isolation, identification, and phylogenetic analyses of endophytic fungi

Isolation of the endophytic fungi was performed based on the procedures described by Xu *et al.* (2008). The cleaned samples were cut into about 5 mm×5 mm×5 mm cubes and then surface-disinfected by washing in 75% ethanol for 1 min, sterile distilled water twice, 0.05 g/ml sodium hypochlorite solution for 3 min followed by two rinses in sterile distilled water. The surface-sterilized samples were cut into small pieces using a sterile blade and placed on plates with potato-dextrose agar (PDA) medium (the medium contained potato 200 g, glucose 20 g, and agar 15 g in 1 L of purified water) for incubation at 25 °C. Each fungus

from plant tissue was removed and placed onto a new PDA petri plate until cultures were obtained for identification and fermentation.

Fungal identification methods were based on their internal transcribed spacer ribosomal DNA (ITS-rDNA) sequences. A pair of primers ITS1 (sequence: 5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') was used for ITS-rDNA amplification (Phongpaichit *et al.*, 2006). The corresponding ITS-rDNA sequence of each endophytic fungus was then used for similarity analysis using BlastN algorithm against the public database at the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov>). All of the fungal ITS-rDNA sequences were deposited in GenBank (accession Nos. GU355645–GU355672). Multiple sequence alignments were performed using the CLUSTAL X program (Thompson *et al.*, 1994) and molecular evolutionary analyses were conducted using MEGA Version 4.0 (Kumar *et al.*, 2008). The Kimura (1980)'s two-parameter model was used to estimate evolutionary distance. The phylogenetic tree was constructed using the neighbor-joining (NJ) algorithm (Naruya and Masatoshi, 1987) and maximum-parsimony (MP) analyses, with bootstrap values calculated from 1000 replicate runs using the software routines included in the MEGA software (Li *et al.*, 2008).

### 2.3 Preparation of fungal fermentation broth

The endophytic fungal isolates were cultured in potato dextrose liquid medium (the medium contained potato 200 g and glucose 20 g in 1 L of purified water) for 10 d at 25 °C on a shaker at 180 r/min. Crude fermentation broth was blended thoroughly and centrifuged at 4000 r/min for 5 min. Liquid supernatant was extracted with an equal volume of ethyl acetate thrice. The organic solvent extract was then evaporated under reduced pressure to yield an ethyl acetate extract. The ethyl acetate extracts were dissolved in sterilized water to a final concentration of 0.5 mg/ml for antimicrobial and antitumor activity screening (Lv *et al.*, 2010).

### 2.4 Antimicrobial activity assay

Gram-negative *Escherichia coli* and two Gram-positive species *Bacillus subtilis* and *Staphylococcus aureus* were used as test bacteria. Three pathogenic

fungi *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* were used as indicator microorganisms to determine antifungal activity. All six indicator organisms were obtained from the Chinese Academy of Medical Sciences (CAMS). The agar well diffusion method was then employed to evaluate the antimicrobial activity (Rios *et al.*, 1988). The bacteria were diluted with melted beef extract peptone (BEP) medium (beef extract 5 g, NaCl 5 g, peptone 10 g, and agar 15 g in 1 L of sterilized water; pH 7.2) to give  $1 \times 10^6$  bacteria/ml of bacteria and poured into a 9-cm diameter petri plate containing 8 ml of solidified BEP. The concentration of fungal spores was adjusted to  $1 \times 10^5$  spores/ml by diluting it with melted Sabouraud's agar (SA) medium and poured into a 9-cm diameter petri plate containing 8 ml of solidified SA medium. After solidification, four circular equidistant wells (7.8 mm in diameter) were made in the BEP or SA layer using sterile cork borers. Then 80  $\mu$ l of each fungal extract solution with a final concentration of 0.5 mg/ml was added into the wells. After incubation at 37 °C for 24 h for bacteria or at 25 °C for 48 h for fungi, the inhibition zones were observed, measured, and recorded. All tests were performed in triplicate.

## 2.5 Antitumor activity assay

Five human cancer lines (HepG2, MCF7, SKVO3, HL-60, and 293-T) were obtained from CAMS. HepG2, MCF7, HL-60, and 293-T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mmol L-glutamine, 100 U/ml of penicillin, and 100  $\mu$ g/ml streptomycin. The SKVO3 cell line was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mmol/L (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.0 mmol/L sodium pyruvate, 10% fetal bovine serum, 100 U/ml of penicillin, and 100  $\mu$ g/ml streptomycin. The tumor cell cultures were maintained at 37 °C in an atmosphere of 5% CO<sub>2</sub> and 95% air with more than 95% relative humidity. Antitumor activity assay was conducted by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay protocol was adapted from that described by Mosmann (1983) and Alley *et al.* (1988). The spectrophotometric absorbance

at 578 nm was measured and the assay was performed in triplicate. Growth inhibition rate (IR) was calculated by the following formula:  $IR = (OD_{con.} - OD_{treated}) / OD_{con.} \times 100\%$ , where OD<sub>con.</sub> and OD<sub>treated</sub> are ODs of control and treated wells, respectively.

## 3 Results

### 3.1 Endophytic fungi and their phylogenetic analyses

A total of 28 morphologically distinct fungal isolates were isolated and identified from the agarwood. They were classified into 14 genera, according to the results of molecular identification (Tables 1 and 2). Among the fungi isolated, 15 were from the agarwood collected at Yunnan and belonged to 12 different genera, 13 were from Hainan and belonged to 5 genera (Table 1). *Fusarium* and *Phaeoacremonium* were the dominant genera. *Fusarium*, *Phaeoacremonium*, and *Phoma* colonized both Yunnan agarwood and Hainan agarwood, while *Chaetomium* sp. and *Pichia* sp. were only found in Hainan agarwood. Nine other genera were only found in Yunnan agarwood.

**Table 1** Genera of endophytic fungi isolated from agarwood (*A. sinensis*) from Yunnan and Hainan

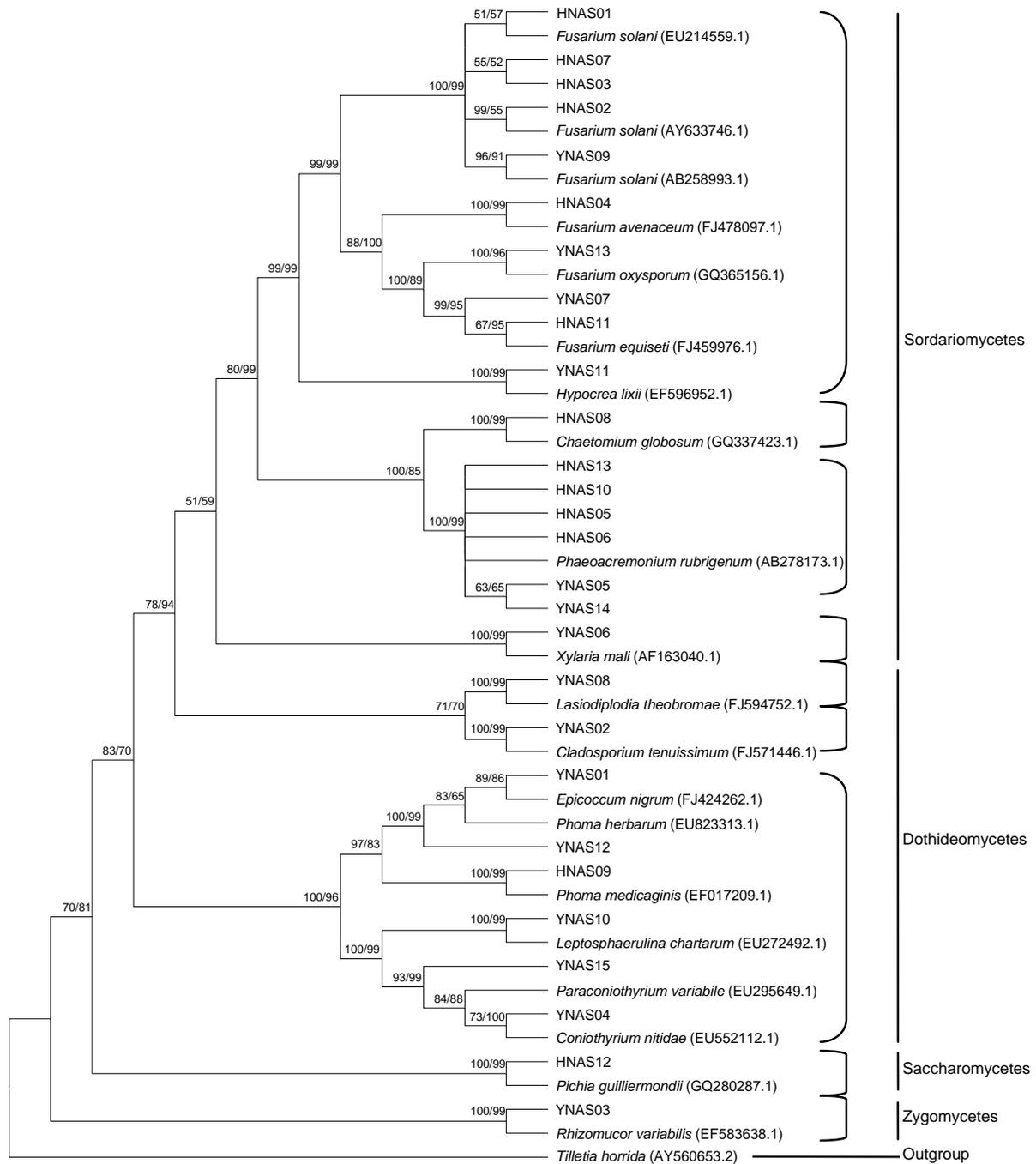
| Genus                   | Number of fungal isolates |        |
|-------------------------|---------------------------|--------|
|                         | Yunnan                    | Hainan |
| <i>Epicoccum</i>        | 1                         | 0      |
| <i>Cladosporium</i>     | 1                         | 0      |
| <i>Rhizomucor</i>       | 1                         | 0      |
| <i>Paraconiothyrium</i> | 1                         | 0      |
| <i>Phaeoacremonium</i>  | 2                         | 4      |
| <i>Xylaria</i>          | 1                         | 0      |
| <i>Fusarium</i>         | 3                         | 6      |
| <i>Lasiodiplodia</i>    | 1                         | 0      |
| <i>Leptosphaerulina</i> | 1                         | 0      |
| <i>Hypocrea</i>         | 1                         | 0      |
| <i>Phoma</i>            | 1                         | 1      |
| <i>Coniothyrium</i>     | 1                         | 0      |
| <i>Chaetomium</i>       | 0                         | 1      |
| <i>Pichia</i>           | 0                         | 1      |

Aside from *Rhizomucor variabilis* (Zygomycota) all the endophytes were Ascomycota or anamorphic fungi that belonged to three classes (Sordariomycetes, Dothideomycetes, and Saccharomycetes) (Fig. 1).

Table 2 Antimicrobial and cytotoxic activities of endophytic fungi from agarwood (*A. sinensis*)

| Code   | Closest identified relative                    | Similarity (%) | Antimicrobial activity <sup>a</sup> |      |     |    |    |    | Cytotoxic activity <sup>b</sup> |      |       |       |       |  |
|--------|--|----------------|-------------------------------------|------|-----|----|----|----|---------------------------------|------|-------|-------|-------|--|
|        |  |                | EC                                  | BS   | SA  | CA | CN | AF | HepG2                           | MCF7 | SKVO3 | HL-60 | 293-T |  |
| YNAS01 | <i>Epicoccum nigrum</i> (FJ424262.1)           | 100            | -                                   | -    | -   | -  | -  | -  | *                               | **   | -     | -     | *     |  |
| YNAS02 | <i>Cladosporium tenuissimum</i> (FJ571446.1)   | 100            | -                                   | -    | -   | -  | -  | -  | **                              | **   | -     | -     | -     |  |
| YNAS03 | <i>Rhizomucor variabilis</i> (EF583638.1)      | 100            | -                                   | -    | -   | -  | ++ | -  | **                              | **   | -     | -     | -     |  |
| YNAS04 | <i>Paraconiothyrium variabile</i> (EU295649.1) | 100            | -                                   | -    | -   | -  | -  | -  | -                               | -    | -     | -     | -     |  |
| YNAS05 | <i>Phaeoacremonium rubrigenum</i> (AB278173.1) | 100            | -                                   | -    | -   | -  | -  | -  | **                              | -    | -     | -     | -     |  |
| YNAS06 | <i>Xylaria mali</i> (AF163040.1)               | 100            | -                                   | -    | -   | -  | -  | -  | -                               | ***  | ***   | ***   | ****  |  |
| YNAS07 | <i>Fusarium equiseti</i> (FJ459976.1)          | 100            | -                                   | -    | +++ | -  | -  | -  | -                               | **   | -     | -     | **    |  |
| YNAS08 | <i>Lasiodiplodia theobromae</i> (FJ594752.1)   | 100            | -                                   | -    | -   | -  | -  | -  | -                               | **   | *     | -     | ****  |  |
| YNAS09 | <i>Fusarium solani</i> (AB258993.1)            | 100            | -                                   | +    | +   | +  | -  | -  | *                               | **   | -     | -     | *     |  |
| YNAS10 | <i>Leptosphaerulina chartarum</i> (EU272492.1) | 99             | ++                                  | -    | +   | ++ | -  | -  | *                               | ***  | -     | -     | *     |  |
| YNAS11 | <i>Hypocrea lixii</i> (EF596952.1)             | 100            | -                                   | -    | -   | -  | -  | -  | -                               | **   | -     | -     | *     |  |
| YNAS12 | <i>Phoma herbarum</i> (EU823313.1)             | 99             | -                                   | -    | -   | -  | -  | -  | -                               | **   | -     | -     | -     |  |
| YNAS13 | <i>Fusarium oxysporum</i> (GQ365156.1)         | 100            | ++                                  | -    | +   | -  | -  | -  | *                               | **   | -     | -     | **    |  |
| YNAS14 | <i>Phaeoacremonium rubrigenum</i> (AB278173.1) | 100            | +++                                 | -    | -   | +  | -  | -  | -                               | **   | -     | -     | -     |  |
| YNAS15 | <i>Coniothyrium nitidae</i> (EU552112.1)       | 95             | ++                                  | -    | +   | +  | -  | -  | *                               | ***  | -     | -     | -     |  |
| HNAS01 | <i>Fusarium solani</i> (EU214559.1)            | 100            | -                                   | -    | -   | -  | -  | -  | *                               | -    | -     | -     | -     |  |
| HNAS02 | <i>Fusarium solani</i> (AY633746.1)            | 99             | -                                   | -    | -   | -  | -  | -  | -                               | **   | *     | -     | -     |  |
| HNAS03 | <i>Fusarium solani</i> (EU214559.1)            | 100            | -                                   | -    | -   | -  | -  | -  | -                               | -    | *     | -     | -     |  |
| HNAS04 | <i>Fusarium avenaceum</i> (FJ478097.1)         | 99             | +                                   | +++  | -   | +  | -  | -  | *                               | **   | -     | -     | **    |  |
| HNAS05 | <i>Phaeoacremonium rubrigenum</i> (AB278173.1) | 100            | -                                   | +++  | -   | +  | -  | -  | *                               | **   | -     | -     | *     |  |
| HNAS06 | <i>Phaeoacremonium rubrigenum</i> (AB278173.1) | 100            | -                                   | -    | -   | -  | -  | -  | -                               | **** | -     | -     | -     |  |
| HNAS07 | <i>Fusarium solani</i> (EU214559.1)            | 100            | -                                   | -    | -   | -  | -  | -  | **                              | *    | *     | -     | ***   |  |
| HNAS08 | <i>Chaetomium globosum</i> (GQ337423.1)        | 100            | -                                   | -    | -   | ++ | -  | -  | *                               | **   | -     | -     | **    |  |
| HNAS09 | <i>Phoma medicaginis</i> (EF017209.1)          | 100            | -                                   | -    | -   | -  | -  | -  | -                               | ***  | -     | -     | -     |  |
| HNAS10 | <i>Phaeoacremonium rubrigenum</i> (AB278173.1) | 100            | -                                   | +    | +   | -  | -  | -  | -                               | -    | -     | -     | -     |  |
| HNAS11 | <i>Fusarium equiseti</i> (FJ459976.1)          | 100            | -                                   | ++++ | -   | -  | -  | -  | -                               | -    | -     | -     | -     |  |
| HNAS12 | <i>Pichia guilliermondii</i> (GQ280287.1)      | 100            | -                                   | -    | -   | -  | -  | -  | -                               | -    | -     | -     | -     |  |
| HNAS13 | <i>Phaeoacremonium rubrigenum</i> (AB278173.1) | 100            | -                                   | -    | -   | ++ | -  | -  | -                               | -    | -     | -     | -     |  |

EC: *Escherichia coli*; BS: *Bacillus subtilis*; SA: *Staphylococcus aureus*; CA: *Candida albicans*; CN: *Cryptococcus neoformans*; AF: *Aspergillus fumigatus*. <sup>a</sup>The antimicrobial activity is expressed by the diameter (d) of inhibition zone: -, d<8.0 mm; +, 8.0 mm≤d<11.0 mm; ++, 11.0 mm≤d<14.0 mm; +++, 14.0 mm≤d<17.0 mm; +++++, d≥17.0 mm. <sup>b</sup>The cytotoxic activity is expressed by the inhibition rate (IR): -, IR<10%; \*, 10%≤IR<20%; \*\*, 20%≤IR<40%; \*\*\*, 40%≤IR<60%; \*\*\*\*, 60%≤IR<80%; \*\*\*\*\*, 80%≤IR<80%; IR≥80%.



**Fig. 1** Neighbour-joining (NJ) phylogenetic tree based on ITS-rDNA sequences of 28 endophytic fungi isolated from the agarwood (*A. sinensis*) and the closest identified relatives from GenBank

Numbers above branches indicate bootstrap values of NJ/MP (>50%, right) from 1000 bootstrap replicates. Isolates YNAS came from Yunnan Province and isolates HNAS came from Hainan Province

Sordariomycetes, the biggest group, contained 18 independent isolates that belonged to four orders (Hypocreales, Sordariales, Calosphaerales, Xylariales) and five genera (*Fusarium*, *Hypocrea*, *Chaetomium*, *Phaeoacremonium*, and *Xylaria*). Eight

isolates spanning seven genera (*Lasiodiplodia*, *Cladosporium*, *Epicoccum*, *Phoma*, *Leptosphaerulina*, *Paraconiothyrium*, and *Coniothyrium*) belonged to the Dothideomycetes. This class contained three taxonomic orders (Botryosphaerales, Capnodiales,

Pleosporales). Isolate HNAS12, sharing 100% similarity with *Pichia guilliermondii*, was the only member of the Saccharomycetes group, and it formed subgroup 8, corresponding to the order Saccharomycetales. Isolate YNAS03 formed subgroup 9 with *Rhizomucor variabilis* and belonged to the Zygomycetes (Fig. 1).

### 3.2 Antimicrobial activity

The fermentation broths of the 28 endophytic fungal isolates from agarwood were screened for antimicrobial activity against six microbial pathogens by the agar well diffusion method (Table 2). Thirteen isolates (46.4%) showed promising growth inhibitory activity against at least one of the test strains, but no endophyte had antimicrobial activity against all six pathogenic microbes (Table 2). A high proportion of fungi (28.6%) had activity against *C. albicans*. The numbers of fungal isolates displaying antimicrobial activity against *E. coli*, *B. subtilis*, *S. aureus*, and *A. fumigatus* were five, five, six, and two, respectively. No isolate displayed antagonistic activity against *C. neoformans*. YNAS14 showed promising growth inhibitory activity against *E. coli*. The isolate HNAS11 exhibited a high antimicrobial activity against *B. subtilis*. HNAS04 and HNAS05 showed a similar, but slightly lower than HNAS11, degree of antagonistic activity against *B. subtilis*. Isolate YNAS07 was most high antagonistic to *S. aureus*, while HNAS08 had a high inhibitory activity against *A. fumigatus*.

### 3.3 Antitumor activity

The fermentation broths from the 23 fungi displayed antitumor activity against at least one of five cancer cell lines. Fourteen were from the agarwood of Yunnan and nine were from Hainan (Table 2). Among them, 7 isolates (25.0%) displayed activity against HepG2 cells, 11 (39.3%) against MCF7 cells, 19 (67.9%) against SKVO3, 5 (17.9%) against HL-60 cells, and 12 (42.9%) against 293-T cells. Therefore, the highest proportion of active isolates was that from the test of SKVO3 cells, followed by 293-T, and MCF7. In contrast, all isolates showed low cytotoxic effect toward HepG2 and HL-60. The isolates YNAS06 and YNAS08 displayed a high antitumor activity on 293-T, and the isolate HNAS06 showed a high antitumor activity on SKVO3 cells. They may have potential practical value.

## 4 Discussion

Endophytes are presumably ubiquitous in plants, with populations dependent on host species and location (Tan and Zou, 2001). Not all endophytes may have been isolated since some may not grow under laboratory conditions, some may grow too slowly to be seen, and some fungi may be missed because their morphology characteristics were very similar to others. Endophytes from a particular host usually include one to several taxa that are adapted to that host (Schulz and Boyle, 2005). For example, *Discula umbrinella* is primarily found in *Fagus sylvatica* (Sieber and Hugentobler, 1987), *Physalospora vaccinii* in *Vaccinium oxycoccus* (Schulz et al., 1993), and in current study more than 50% of the isolates were *Fusarium* spp. and *Phaeoacremonium rubigenum*-like isolates.

It was reported that endophytic fungi were isolated from the leaves, stems, and roots of *A. sinensis* (Gong and Guo, 2009; Wang et al., 2009). Gong and Guo (2009) found that the dominant genus was *mycelia sterilia* sp., and Wang et al. (2009) reported that it was *Colletotrichum* sp. However, the results of the current study indicated that the dominant genus was *Fusarium* sp. from agarwood, which indicated that the composition and structure of fungal species in agarwood were different to those that do not produce fragrant ingredients in *A. sinensis*.

Gong and Guo (2009) obtained four isolates (3.1%) which could inhibit at least one of the tested fungi and bacteria by the same screening process of 128 endophytic fungi from healthy tissues of *A. sinensis*. The inhibition rates of the above endophytic fungi were lower than those from agarwood in the present research.

The bioactive abilities of endophytic fungi were not identical, even though they were isolated from the same host species, and belonged to the same genus. For example, *Fusarium solani* (YNAS09) displayed antimicrobial activity to *B. subtilis*, *S. aureus*, and *C. albicans*, and showed antitumor activity against SKOV3. On the other hand, *F. solani* (HNAS07) had no antimicrobial or antitumor activity against SKOV3, but displayed antitumor activity against the other four tumor cells. The other example was that for both YNAS05 and HNAS05 belonging to *Phaeoacremonium rubrigenum*, the former displayed

no antimicrobial activity and showed cytoactivity against HepG2 and MCF7 cells, while the latter had antimicrobial activity to *B. subtilis* and *C. albicans*, but inhibited growth of HepG2, MCF7, SKVO3, and 293-T cells. These results demonstrated that those endophytic fungi with diverse bioactivities in the study were potential drug candidates. We are currently undertaking research on naturally bioactive chemicals putatively produced by the endophytic fungi identified in the present study. In the future, studies should focus on whether the endophytes attribute to the formation mechanism of agarwood in *A. sinensis*.

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