Evaluation of the antimicrobial and cytotoxic potential of endophytic fungi extracts from mangrove plants Rhizophora stylosa and R. mucronata

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Mangrove endophytic fungi are tolerant to numerous stresses and are inevitably capable of exhibiting excellent biological activity by producing impressive numbers of metabolites with special biological functions, based on previous work on the biological potential of mangrove-derived endophytic fungi. To obtain marked antimicrobial and cytotoxic fermentation products of culturable endophytic fungi from mangrove forests, our research evaluated the antimicrobial and cytotoxic activities of crude extracts of endophytic fungi from Rhizophora stylosa and Rhizophora mucronata. Forty-six fungal isolates were cultured on four different media, namely, dextrose agar (PDA), Czapek’s agar (CZA), rice medium (RM) and grain medium (GM) and harvested by ethyl acetate solvent at 40 days. The extracts were tested for antimicrobial activity by the microdilution method against the gram-negative bacteria Pseudomonas adaceae (PA), gram-positive bacteria Enterococcus faecalis (EF), methicillin-resistant Staphylococcus aureus (MRSA) and pathogenic fungus Monilia albicans (MA). The cytotoxic activity of the extracts was evaluated by MTT assay using A549 human lung cancer cells, HeLa human cervical carcinoma cells, and HepG2 human hepatocellular cells. The results showed that rice medium could promote the secretion of antimicrobial and antitumour secondary metabolites of endophytic fungi in comparison with other cultivation media. Seventeen strains (68%) from R. stylosa exhibited inhibitory effects on indicators, especially N. protearum HHL46, which could inhibit the growth of four microbes with MIC values reaching 0.0625 mg/mL. Fifteen strains (71.4%) from R. mucronata displayed activities against human pathogenic microbes; in particular, Pestalotiopsis sp. HQD6 and N. protearum HQD5 could resist the growth of four microbes with MIC values ranging from 0.015 to 1 mg/mL. In the cytotoxicity assay, the extracts of 10 strains (40%), 9 strains (40%) and 13 strains (52%) of R. stylosa and 13 strains (61.9%), 10 strains (47.6%) and 10 strains (47.6%) of R. mucronata displayed cytotoxicity against A549, HeLa and HepG2 cancer cells with cell viability values ≤ 50%. Neopestalotiopsis protearum HHL46, Phomopsis longicolla HHL50, Botryosphaeria fusispora HQD83, Fusarium verticillioides HQD48 and Pestalotiopsis sp. HQD6 displayed significant antitumour activity with IC50 values below 20 μg/mL. These results highlighted the antimicrobial and antitumour potential of endophytic fungi from R. stylosa and R. mucronata and the possibility of exploiting their antimicrobial and cytotoxic agents.

For decades, natural products have been among the most successful sources of drugs to treat infectious diseases, such as penicillin, vancomycin and daptomycin. However, their indiscriminate use has led to a relentless and pernicious emergence of antimicrobial resistance to all major classes of these drugs and the emergence of new and reemergent infectious diseases. As a consequence, there is an alarming scarcity of new antibiotic classes in the pipelines of the pharmaceutical industry. Cancer is the second leading cause of death worldwide, and the mortality of the common forms of cancer is unacceptably high. Conventional therapies cause serious side effects and, at best, merely extend the patient’s lifespan by a few years. The mortality statistics are unlikely to

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change until there is a reorientation of concepts for the use of natural products as new chemopreventive agents. Hence, naturally derived compounds have received increasing attention in the past 40 years for the discovery of novel cancer preventive and therapeutic agents. Over 60% of clinically useful anticancer agents and a considerable number of natural products or analogously derived therapeutics are in clinical and preclinical development. Examples include taxanes, Vinca alkaloids, podophyllotoxins and camptothecin. The anticancer drug potential of microbes remains relatively unexplored, but it is becoming increasingly evident that the realm of microbes from unusual or specialized ecological niches, such as mangrove habitats, offers vast untapped potential.

Mangrove forests are complex ecosystems that are distributed in the intertidal zone of tropical and subtropical coasts, which not only refers to mangrove plants but also includes diverse groups of microorganisms formed by abundant biological communities. There are 61 species of true mangrove plants in the world, belonging to 14 families and 21 genera. China has 26 species, and 24 of them have been documented in Hainan. Mangrove endophytic fungi tolerant of numerous stresses, such as high salinity, high temperature, extreme tides, oxygen pressure, high humidity, and light and air limitations, have evolved unique metabolic pathways for the purposes of competition over nutrition and space in extremely fierce niches, which will inevitably render them capable of exhibiting excellent biological activity by producing impressive numbers of metabolites with special biological functions. Rivali et al. studied the antimicrobial activities of endophytic fungi from *R. mucronata*, and ethyl acetate extracts of 14 strains (64.3%) showed activity against the test microbes *S. aureus, E. coli*, and *C. albicans*. *Cladosporium* sp. isolated from *Thepesia populneoides* and *Xylaria* sp. isolated from *Acanthace (Acanthus ilicifolius)*, *Xylospora (Xylospora naticae)*, and *Malacca (Thepesia populneoides)* and showed cytotoxicity against some cancer cell lines, including A375 (human malignant melanoma), SW620 (human colorectal adenocarcinoma), Kato III (human gastric carcinoma), HepG2 (human liver hepatoblastoma) and Jurkat (human acute T cell leukemia) (Table 1). Extracts of 9 endophytic fungal isolates (64.3%) from *R. mucronata* can inhibit the growth of the tested bacteria and fungi, of which 12 isolates (85.7%) were cytotoxic (cell viability < 50%) against T47D cells.

*Rhizophora* is one of the most conspicuous genera of the most widespread mangrove family, Rhizophoraceae. Sixty-six endophytic fungi were isolated from plants of the genus *Rhizophora*, such as *Aspergillus, Cladosporium, Chaetomium, Fusarium, Lasiodiplodia, Penicillium, Pestalotiopsis, Phomopsis, Phoma, Phyllotisctica, and Trichoderma*, more than 195 natural products, including alkaloids, terpenoids, coumarins, chromones, quinones, peptides, phenolic acids, and lactones, were identified, and cytotoxicity was found to be the most notable bioactivity of the secondary metabolites isolated. Among them, unprecedented scaffolds of diterpenoids were isolated from *Mucor irregularis* QEN-189 in *R. stylosa* demonstrated activity against human cancer HL-60 and A-549 cell lines with an IC₅₀ value ranging from 5 to 15 μM and are novel inhibitors of the Wnt/β-catenin pathway in breast cancer cells. A novel cytochalasin, which was found in the endophytic fungus *Phomopsis* sp. derived from *Kandelia candel*, can effectively induce apoptosis and inhibit the migration of A549 human lung cancer cells by significantly increasing the protein expression of Bax, p53 and cleaved Caspase-3 and increasing the ratio of the antiapoptotic proteins Bax/Bcl-2. In addition, demethylincosterol A₃ was a selective inhibitor of the classical nonreceptor protein tyrosine phosphatase Sph2 and isolated from the endophytic *F. nigricans* *Pestalotiopsis* sp. HQD619. Two new polyketide derivatives pestalpol and pestalin were obtained from extracts of the endophytic fungus *P. clavigora* isolated from *R. harrisonii* and exhibited strong cytotoxicity against the mouse lymphoma cell line L5178Y with an IC₅₀ value of 4.10 μM.

During our previous work on the biological potential of mangrove-derived endophytic fungi, we isolated and identified endophytic fungi from mangroves *R. stylosa* and *R. mucronata*, and their antioxidant activities were evaluated. In this study, we continuously investigated the antimicrobial and cytotoxic activities of endophytic fungi isolated from *R. stylosa* and *R. mucronata*. We tried to promote the secretion of antibacterial and antitumor substances of our fungal isolates by using four different culture media. Four microbial indicator strains (PA, EF, MRSA and MA) and three human cancer cell lines (HeLa, A549 and HepG2) were adopted for antimicrobial and cytotoxicity tests, respectively. This study aims to provide constructive information on the in vitro potential of the endophytic fungi of these two hosts as producers of antimicrobial and cytotoxic activities.

**Results**

**Antimicrobial activity of fungal extracts.** From a total of 46 fungal extracts assayed, 32 extracts (69.6%) showed antimicrobial activity against at least one of the indicator pathogenic microbes tested (Tables 1 and 2). The antimicrobial activity of the same isolated fungal strain was significantly different (P < 0.05) when cultured on different media, but all of the antimicrobial activities were weaker than that of the positive control.

Of the endophytic fungi isolated from *R. stylosa* (25 isolates, Table 1), RM was determined to be more suitable for antibiotic production in fungal isolates than the other three media (P < 0.05). Of these, 13 strains cultured on RM (52%) exhibited antimicrobial activity using 1 mg/mL extracts, and among them, 7 strains had stronger inhibitory effects on MRSA with MIC values less than 5 μg/mL. HHL55 showed the broadest antimicrobial spectrum against four indicator test microorganisms, and CZA culture of HHL55 was found to show the most potent antimicrobial activity against PA with an MIC value of 0.031 mg/mL. Only 2 strains fermented on the GM displayed low inhibitory activity with an MIC value of 1 mg/mL. In addition, three culture media derived from HHL94, HHL64 and HHL82 showed inhibitory effects on three indicator microorganisms.

Of the endophytic fungi isolated from *R. mucronata* (21 isolates, Table 2), most of the fungal strains (15 isolates, 71.4%) exhibited antimicrobial activity. A high growth inhibition rate was detected from the fungal extracts of the endophytic fungi of these two hosts as producers of antimicrobial and cytotoxic activities.
extracts cultured on CZA (10 isolates, 47.6%) and RM (10 isolates, 47.6%) in comparison with PDA (7 isolates, 33.3%) and GM (2 isolates, 9.5%) at the selected concentration of 1 mg/mL (P < 0.05). The RM of 9 isolates and CZA of 3 isolates showed strong inhibitory effects on MRSA with MIC values less than 0.5 mg/mL. Only the extract of HQD20 cultivated on GM inhibited the growth of MRSA and MA. Moreover, HQD6 and HQD5 displayed antimicrobial activity against the growth of four indicator microorganisms tested with MIC values ranging from 0.015 to 1 mg/mL.

Cytotoxicity of fungal extracts. The cytotoxic effects of *R. stylosa* and *R. mucronata* endophytic fungal extracts were tested against HeLa, A549 and HepG2 cells using an MTT colorimetric assay. The half inhibitory concentration (IC_{50}) values are shown in Tables 3 and 4. The results are presented as the means ± standard deviation of experiments performed in triplicate. Doxorubicin was used as the positive control, and the IC_{50} values were 0.17 ± 0.01 nM, 8.6 ± 0.1 nM and 137 ± 0.9 nM for A549, HeLa and HepG2 cells, respectively, which

| Number       | Species                              | MIC mg/mL |
|--------------|--------------------------------------|-----------|
|              |                                      | PDAa      | CZA       | RM         | GM         |
|              |                                      | M.A5      | MRSA P.A  | E.F        | M.A5       | MRSA P.A  | E.F        | M.A5       | MRSA P.A  | E.F        | M.A5       | MRSA P.A  | E.F        |
| HHL70        | *Botryosphaeria dothidea*            | 1         | 1         | –         | –          | –          | –          | –          | –          | –          | –          | –          | –          |
| HHL104       | *Cladosporium cladosporioides*       | –         | –         | –         | –          | –          | –          | –          | –          | –          | –          | –          | –          |
| HHL55        | *Cytopora rhizophorae*               | –         | 1         | –         | 0.0625     | 0.031      | –          | 1          | 1          | –          | 1          | 1          |
| HHL59        | *Diaperthe ceratozamiae*             | –         | –         | –         | –          | –          | –          | –          | 1          | –          | –          | –          | –          |
| HHL53        | *Diaperthe eucalyptorum*             | –         | –         | –         | –          | 1          | –          | 1          | 1          | –          | –          | –          | –          |
| HHL61        | *Diaperthe persee*                   | –         | –         | –         | –          | –          | –          | 1          | 1          | –          | –          | –          | –          |
| HHL7         | *Diaperthe sp.*                      | –         | –         | –         | 1          | –          | –          | –          | –          | –          | –          | –          | –          |
| HHL48        | *Fusarium solani*                    | 1         | –         | –         | –          | –          | –          | –          | –          | –          | –          | –          | –          |
| HHL96        | *Guignardia mangiferae*              | –         | –         | –         | –          | –          | –          | –          | –          | –          | –          | –          | –          |
| HHL31        | *Lasiodiplodia pseudochrombora*      | –         | –         | –         | –          | –          | –          | –          | –          | –          | –          | –          | –          |
| HHL94        | *Lasiodiplodia theobromae*           | 1         | 0.125     | 0.125     | 0.125      | –          | –          | –          | –          | –          | –          | –          | –          |
| HHL129       | *Neofusicoccum mangiferae*           | –         | 1         | –         | –          | –          | –          | –          | 0.25       | –          | –          | –          | –          |
| HHL75        | *Neofusicoccum parvum*               | –         | –         | –         | –          | –          | –          | –          | 0.25       | –          | –          | –          | –          |
| HHL46        | *Neopatalotiopsis protean*            | –         | 1         | 1         | 0.0625     | 0.062      | 1          | 0.125      | 0.5        | –          | –          | –          | –          |
| HHL82        | *Neopatalotiopsis microspora*        | –         | –         | –         | 1          | 0.125      | 0.125      | 0.5        | 1          | 1          | 1          | –          | –          |
| HHL51        | *Neopatalotiopsis palarum*           | –         | –         | –         | –          | –          | –          | –          | –          | –          | –          | –          | –          |
| HHL79        | *Neopatalotiopsis phasinia*          | –         | –         | –         | –          | –          | –          | –          | –          | –          | –          | –          | –          |
| HHL10        | *Neopatalotiopsis sp.*               | –         | –         | –         | –          | –          | –          | –          | –          | –          | –          | –          | –          |
| HHL56        | *Neopatalotiopsis theae*             | –         | –         | –         | –          | –          | –          | –          | –          | –          | –          | –          | –          |
| HHL22        | *Phomopsis asparagi*                 | –         | –         | –         | –          | –          | 1          | –          | –          | –          | –          | –          | –          |
| HHL52        | *Phomopsis glabrea*                  | –         | –         | –         | –          | –          | –          | –          | –          | –          | –          | –          | –          |
| HHL50        | *Phomopsis longicolla*               | –         | –         | 1         | 1          | –          | –          | 1          | 0.25       | –          | –          | –          | –          |
| HHL20        | *Phomopsis sp.*                      | –         | –         | –         | 1          | 1          | –          | –          | –          | –          | –          | –          | –          |
| HHL38        | *Seiridium ceratozamiae*             | –         | –         | –         | –          | –          | –          | –          | 0.5        | –          | –          | –          | –          |
| HHL81        | *Valsa brevispora*                   | –         | –         | –         | –          | 1          | 0.25       | –          | –          | –          | –          | –          | –          |
| Ciprofloxacin (μg/mL) |              | 0.16      | 0.08      | 0.63      | –          | –          | –          | –          | –          | –          | –          | –          | –          |
| Amphotericin B (μg/mL)   |                      | 0.04      | –         | –         | –          | –          | –          | –          | –          | –          | –          | –          | –          |

Table 1. Antimicrobial activity of the endophytic fungi from *R. stylosa*. aStrains were cultivated on four different medium, which were Dextrose Agar (PDA), Czapek’s Agar (CZA), Rice Medium (RM) and Grain Medium (GM) from *R. stylosa*. bAntimicrobial activities were tested against Gram-negative [*Pseudomonas adaeae* (PA)], Gram-positive [*Enterococcus faecalis* (EF)], Methicillin-resistant *Staphylococcus aureus* (MRSA)] bacteria and fungi [*Monilia albican* (MA)].
were stronger than the cytotoxicity of the fungal extracts. Cell viability using different concentrations of these extracts after 24 h of treatment was determined (Figs. 1 and 2). The cytotoxicity of fungal extracts showed a dose-dependent relationship.

As shown in Table 3, of the endophytic fungi isolated from R. stylosa, extracts of 10 isolates (40%) tested were cytotoxic and exhibited a viability percentage of A549 cells ≤ 50%, with all of the Phomopsis sp. and 3 isolates (50% of the Pestalotiopsis sp.) showing antitumour activity, and the IC50 values of four extracts (HHL75, HHL46, HHL10 and HHL50) were lower than 100 μg/mL (IC50 values of 64.38 ± 3.40, 11.65 ± 0.34, 97.21 ± 1.36 and 16.31 ± 0.36 μg/mL, respectively), suggesting the cytotoxic potential of these four fungal isolates; 75% of them belong to Phomopsis sp. and Pestalotiopsis sp. (Fig. 1A–D). Extracts of 9 isolates (36%) showed cytotoxicity and displayed a HeLa cell viability percentage ≤ 50%. In particular, the HHL46, HHL82, HHL52 and HHL50 extracts showed the most significant cytotoxic effect, with IC50 values of 31.03 ± 1.21, 14.38 ± 1.84, 70.55 ± 1.37 and 73.18 ± 1.64, respectively, which were lower than 100 μg/mL. The four fermentation products that inhibited HeLa cells were all from Phomopsis sp. and Pestalotiopsis sp., which coincided with the inhibition of A549 cells (Fig. 1E–H). Extracts of 13 isolates (52%) showed cytotoxicity against HepG2 cells, among which 7 extracts (HHL61, HHL75, HHL46, HHL10, HHL52 and HHL50) could significantly suppress the proliferation of HepG2 cells with IC50 values below 100 μg/mL. Eight of the 13 isolates (61.54%) belong to Phomopsis sp. and Pestalotiopsis sp. (Fig. 1I–K). Phomopsis sp. and Pestalotiopsis sp. were the dominant fungi (41.67%) in R. stylosa, with 9 strains inhibiting at least one tumour cell, suggesting that these two fungal genera could have high potential as producers of antitumour compounds.

Table 2. Antimicrobial acticity of the endophytic fungi from R. mucronata. a Strains were cultivated on four different medium, which were Dextrose Agar (PDA), Czapek’s Agar (CZA), Rice Medium (RM) and Grain Medium (GM) from R. mucronata. b Antimicrobial activities were tested against Gram-negative [Pseudomonas adaecea (PA)], Gram-positive [Enterococcus faecalis (EF), Methicillin-resistant Staphylococcus aureus (MRSA)] bacteria and fungi [Monilia albican (MA)].

| Number | Species | MIC mg/mL | PDAa | CZA | RM | GM |
|--------|---------|-----------|------|-----|----|----|
| HQD24  | Aspergillus fumigatus | – | – | – | – | – |
| HQD83  | Botryosphaeria  | – | – | – | – | – |
| HQD25  | Colletotrichum gloeosporioides | – | 1 | – | – | – |
| HQD62  | Diaporthaceae  | – | – | – | 1 | – | 1 |
| HQD33  | Diaporthaceae  | 1 | – | – | – | – | – |
| HQD17  | Diaporthaceae  | – | – | – | 0.062 | 1 | 1 |
| HQD29  | Diaporthaceae  | – | – | – | 1 | – | 0.25 |
| HQD28  | Eutypella scoparia | – | – | – | – | – | – |
| HQD48  | Fusarium verticilloides | – | – | – | 0.062 | – | 0.5 |
| HQD72  | Lastiodiplodia theobromae | – | 1 | – | 0.125 | – | 0.125 |
| HQD23  | Neofuscosciadium mangiferae | – | 1 | – | – | – | – |
| HQD41  | Neofuscosciadium parvum | – | – | – | – | – | – |
| HQD5   | Neofuscosciadium proteus | – | – | 1 | – | 0.062 | – |
| HQD55  | Parasiticulosciadium  | – | – | 1 | 0.125 | – | 0.125 |
| HQD20  | Pestalotiopsis microspora | – | – | – | 1 | – | 0.125 |
| HQD1   | Pestalotiopsis proteus | – | – | – | 1 | – | 0.125 |
| HQD6   | Pestalotiopsis sp. | 1 | 1 | – | 0.015 | 1 | – |
| HQD57  | Phomopsis glabrae | – | – | – | – | – | – |
| HQD8   | Phomopsis longicolla | – | – | – | 1 | 1 | – | 1 |
| HQD47  | Pseudofuscosciadium  | – | – | – | – | – | – |
| HQD22  | Valsa brevispora | – | – | – | – | – | – |
| Ciprofloxacin (µg/mL) | 0.16 | 0.08 | 0.63 | – | – | – | – |
| Amphotericin B (µg/mL) | 0.04 | – | – | – | – | – | – |
## Table 3. Anti-tumor activity of endophytic fungi from *R. stylosa*.

As shown in Table 4, of the endophytic fungi isolated from *R. mucronata*, extracts of 13 isolates (61.9%) displayed cytotoxic activity on A549 cells, 10 isolates (47.6%) on HeLa cells, and 10 isolates (47.6%) on HepG2 cells. Of these, 9 extracts (HQD83, HQD33, HQD28, HQD48, HQD41, HQD5, HQD6, HQD58, HQD81) exhibited significant cytotoxic activity against A549 cells with IC<sub>50</sub> values less than 100 μg/mL (Fig. 2A–D). Four extracts (HQD83, HQD48, HQD5 and HQD20) showed strong cytotoxic effects against the HeLa cell line with IC<sub>50</sub> values less than 100 μg/mL (Fig. 2E–H). Seven extracts (HQD83, HQD28, HQD48, HQD41, HQD5, HQD6 and HQD58) were potent (IC<sub>50</sub> < 100 μg/mL) against HepG2 cells (Fig. 2I–L). Diaporthe sp. and Pestalotiopsis sp. were dominant fungi (38.09%) in *R. mucronata*, with all of them displaying at least one tumour cell. However, the three isolates with the strongest ability to inhibit A549, HeLa and HepG2 cells, 1 isolate (HQD28) showed strong cytotoxic effects against the HeLa cell line with IC<sub>50</sub> value of 14.38 ± 1.84 μg/mL, and 2 isolates (HQD83, HQD48, HQD5) showed strong cytotoxic effects against the HepG2 cell line with IC<sub>50</sub> values of 14.38 ± 1.84 μg/mL, 23.17 ± 4.26 μg/mL and 14.38 ± 1.84 μg/mL, respectively. The significant difference analysis showed that the inhibition of HeLa cells by the products of endophytic fungi of *R. stylosa* cultured on PDA was stronger than those cultured under the other three conditions (P < 0.05). No significant difference (P > 0.05) was observed between the cytotoxicity for A549, HeLa and HepG2 cells, with IC<sub>50</sub> values of 137 ± 0.9 μg/mL, respectively.

In an attempt to promote antitumour substance production, four different media were adopted for fungal isolate cultivation to activate biosynthetic silencing of gene expression. We found that extracts of 1 isolate (HQD28) cultured on PDA, 1 isolate (HQD48) cultured on CZA, 3 isolates (HHL82, HQD52 and HQD6) cultured on RM and 4 isolates (HHL46, HQD48, HQD5 and HQD81) cultured on GM exhibited significant antiproliferative activity against at least one of the tested carcinoma cells with IC<sub>50</sub> values less than 100 μg/mL (Fig. 2). Four extracts (HQD83, HQD48, HQD5 and HQD20) showed strong cytotoxic effects against the HeLa cell line with IC<sub>50</sub> values less than 100 μg/mL (Fig. 2E–H). Seven extracts (HQD83, HQD28, HQD48, HQD41, HQD5, HQD6 and HQD58) were potent (IC<sub>50</sub> < 100 μg/mL) against HepG2 cells (Fig. 2I–L). Diaporthe sp. and Pestalotiopsis sp. were dominant fungi (38.09%) in *R. mucronata*, with all of them displaying at least one tumour cell. However, the three isolates with the strongest ability to inhibit A549, HeLa and HepG2 cells, 1 isolate (HQD28) showed strong cytotoxic effects against the HeLa cell line with IC<sub>50</sub> value of 14.38 ± 1.84 μg/mL, and 2 isolates (HQD83, HQD48, HQD5) showed strong cytotoxic effects against the HepG2 cell line with IC<sub>50</sub> values of 14.38 ± 1.84 μg/mL, 23.17 ± 4.26 μg/mL and 14.38 ± 1.84 μg/mL, respectively. The significant difference analysis showed that the inhibition of HeLa cells by the products of endophytic fungi of *R. stylosa* cultured on RM was stronger than those cultured under the other three conditions (P < 0.05). No significant difference (P > 0.05) was observed between the cytotoxicity for A549 of samples from four cultural media having equal degrees at end of 24 h (Table 3). The extracts from CZA culture of *R. mucronata* endophytic HQD48 and RM culture of HQD20 and HQD6 exhibited cytotoxicity towards A549, HeLa and HepG2 cells, with IC<sub>50</sub> values of 137 ± 0.9 μg/mL, respectively (Table 4). The genera *Pestalotiopsis* and *Phomopsis* were demonstrated to be rich sources of antitumour secondary metabolites. Notably, RM culture
of HQD6 exhibited cytotoxic and antiproliferative effects against three tested cancer cell lines with IC50 values ranging from 9.58 to 14.99 μg/mL.

**Profiling of bioactive metabolites by HPLC.** According to the screening for antimicrobial activity and cytotoxicity, we found that 6 endophytic fungi showed strong bioactive abilities: HHL46, HHL50, HQD5, HQD6, HQD83 and HQD48. The difference significance analysis showed that RM was to be the best to produce active metabolites and the product diversity of endophytic fungi on RM was analyzed by HPLC in further. Figure 3 shows the chromatograms of the fermented products of these endophytic fungi at 254 nm. The HPLC analysis results provide a wealth of information, and the hydrophilic compounds were found in the first 10 min. Within 10 to 15 min, the moderately polar components and hydrophobic components were washed out. RM cultures of HQD5 and HQD6 showed more diverse secondary metabolites than other cultures.

**Discussion**

Infectious diseases, including bacterial infections, pose a serious threat to global health and drug resistance. Unfortunately, there has been resistance detected against every antibiotic on the market, and if antibiotic resistance is not mitigated, pathogenic bacteria will once again become one of the leading causes of mortality, with an estimated yearly death toll of ~10 million by 2050. The development of new antimicrobial agents is pointed out as an effective solution to address this problem. As a promising source of diverse and structurally unprecedented bioactive natural products, mangrove-derived endophytic fungi are unquestionably important and continuously attract considerable attention. In our current study, of 46 endophytic fungal strains investigated,

| NO | Species | IC50μg/mL | A549 | CZA | RM | GM | PDA | CZA | RM | GM | PDA | CZA | RM | GM |
|----|---------|-----------|------|-----|----|----|-----|-----|----|----|-----|-----|----|----|
| HQD24 | Aspergillus fumigatus | 179.67 ± 1.21 | – | – | – | – | – | – | – | – | – | – | – | – |
| HQD33 | Trichoderma virens | 23.62 ± 1.96 | – | – | – | – | – | – | – | – | – | – | – | – |
| HQD25 | Colletotrichum gloeosporioides | 14.49 ± 1.71 | – | – | – | – | – | – | – | – | – | – | – | – |
| HQD62 | Diaporthe eucalyptorum | 201.66 ± 1.12 | – | – | – | – | – | – | – | – | – | – | – | – |
| HQD30 | Diaporthe phaseolorum | 109.46 ± 1.25 | – | – | – | – | – | – | – | – | – | – | – | – |
| HQD28 | Epitrichia scabra | 53.81 ± 1.37 | – | – | – | – | – | – | – | – | – | – | – | – |
| HQD48 | Fusarium verticillioides | 547.42 ± 1.65 | – | – | – | – | – | – | – | – | – | – | – | – |
| HQD72 | Lasiodiplodia theobromae | 144.89 ± 1.75 | – | – | – | – | – | – | – | – | – | – | – | – |
| HQD23 | Neofusicoccum mangiferae | 65.38 ± 1.23 | – | – | – | – | – | – | – | – | – | – | – | – |
| HQD9 | Neofusicoccum parvum | 144.89 ± 1.75 | – | – | – | – | – | – | – | – | – | – | – | – |
| HQD55 | Panaeolus xeromophila | 53.81 ± 1.37 | – | – | – | – | – | – | – | – | – | – | – | – |
| HQD20 | Pestalotia microspora | 126.73 ± 1.21 | – | – | – | – | – | – | – | – | – | – | – | – |
| HQD1 | Pestalotias protearum | 40.09 ± 1.39 | – | – | – | – | – | – | – | – | – | – | – | – |
| HQD6 | Pestalotia sp. | 28.90 ± 1.26 | – | – | – | – | – | – | – | – | – | – | – | – |
| HQD7 | Phomopsis glabrae | 0.17 ± 0.01 | 8.6 ± 0.1 | – | – | – | – | – | – | – | – | – | – | – |
| HQD8 | Phomopsis longicolla | 192.97 ± 1.86 | – | – | – | – | – | – | – | – | – | – | – | – |
| HQD47 | Pseudofusicoccum stromaticum | 53.81 ± 1.37 | – | – | – | – | – | – | – | – | – | – | – | – |
| HQD22 | Valsa brevispora | 277.67 ± 1.21 | – | – | – | – | – | – | – | – | – | – | – | – |

**Table 4. Anti-tumor activity of endophytic fungi from R. mucronata.**

of HQD6 exhibited cytotoxic and antiproliferative effects against three tested cancer cell lines with IC50 values ranging from 9.58 to 14.99 μg/mL.
Figure 1. Antitumour activity of endophytic fungi from *R. stylosa*. (A–D) Shows that the fermentation products of endophytic fungi of *R. stylosa* have an inhibition rate for A549 tumour cells in different media, where (A) is obtained under dextrose agar (PDA) culture conditions, (B) is obtained under Czapek’s agar (CZA) culture conditions, (C) is obtained under rice medium (RM) culture conditions and (D) is obtained under grain medium (GM) culture conditions. (E–H) Shows that the fermentation products of endophytic fungi of *R. stylosa* have an inhibition rate for HeLa tumour cells in different media, where (E) is obtained under dextrose agar (PDA) culture conditions, (F) is obtained under Czapek’s agar (CZA) culture conditions, (G) is obtained under rice medium (RM) culture conditions and (H) is obtained under grain medium (GM) culture conditions. (I–K) shows that the fermentation products of endophytic fungi of *R. stylosa* have an inhibition rate for HepG2 tumour cells in different media, where (I) is results under dextrose agar (PDA) culture conditions, (J) is results under Czapek’s agar (CZA) culture conditions, and (K) is results under rice medium (RM) culture conditions.
Figure 2. Antitumour activity of endophytic fungi from *R. mucronata*. (A–D) Shows that the fermentation products of endophytic fungi of *R. mucronata* have an inhibition rate for A549 tumor cells in different media, where (A) is obtained under dextrose agar (PDA) culture conditions, (B) is obtained under Czapek's agar (CZA) culture conditions, (C) is obtained under rice medium (RM) culture conditions and (D) is obtained under grain medium (GM) culture conditions. (E–H) Shows that the fermentation products of endophytic fungi of *R. mucronata* have an inhibition rate for HeLa tumor cells in different media, where (E) is obtained under dextrose agar (PDA) culture conditions, (F) is obtained under Czapek's agar (CZA) culture conditions, (G) is obtained under rice medium (RM) culture conditions and (H) is obtained under grain medium (GM) culture conditions. (I–L) Shows that the fermentation products of endophytic fungi of *R. mucronata* have an inhibition rate for HepG2 tumor cells in different media, where (I) is results under Dextrose Agar (PDA) cultural condition, (J) is results under Czapek's Agar (CZA) cultural condition, (K) is results under Rice Medium (RM) cultural condition, and (L) is results under Grain Medium (GM) cultural condition.
Cytotoxic agents that selectively impact proliferating cells still play an essential role in tumor treatment. In our study, IC₅₀ values below 20 μg/mL and showed more diverse secondary metabolites than other formulations, which can possibly lead to tumor progression, causing a large number of tumor cells to be released and reducing the activity of T, B, and NK lymphocytes in the postoperative period. It has also generated a large body of information that is being harnessed to develop new therapeutic modalities for treating cancer; however, the search for secondary metabolite production by any given microorganism is to combine removing the tumor with anaesthetic agents after surgery. Unfortunately, the surgical process possibly leads to tumor progression, causing a large number of tumor cells to be released and reducing the activity of T, B, and NK lymphocytes in the postoperative period. It has also generated a large body of information that is being harnessed to develop new therapeutic modalities for treating cancer; however, the search for cytotoxic agents that selectively impact proliferating cells still plays an essential role in tumor treatment.

In our study, of 46 endophytic fungal strains investigated, 23 extracts (50%) showed cytotoxicity, 21 extracts (46.6%) were cytotoxic against A549 cells, 16 extracts (34.78%) were cytotoxic against HeLa cells, and 21 extracts (46.6%) were cytotoxic against HepG cells. This was in accordance with previous reports, in which 9 endophytic fungi were successfully obtained from the leaves of Ginkgo biloba. The extracts of isolates J-1, J-2, and J-3 markedly inhibited the proliferation of HeLa cells, promoted their apoptosis and blocked their migration, and 12 isolate extracts (85.7%) derived from mangrove Rhizophora mucronata were cytotoxic (cell viability < 50%) against T47D cells. In our current study, we found that Pestalotiopsis sp. HQD6 displayed significant antitumor activity with IC₅₀ values below 20 μg/mL and showed more diverse secondary metabolites than other formulations, which was in accordance with our previous reports that demethylincisterol A₃ was isolated from the endophytic Pestalotiopsis sp. HQD6 and showed significant in vitro cytotoxicity against the human cancer cell lines HeLa, A549, and HepG with IC₅₀ values ranging from 0.17 to 14.16 nM. It also reported to be a selective inhibitor of the classical nonreceptor protein tyrosine phosphatase Shp2.

Culture-dependent methods have been developed aiming at substantial increases in biologically active secondary metabolite production by any given microorganism. RM was demonstrated to have the highest suitability for antibiotic production; 23 extracts (50%) showed antimicrobial activity against at least one of four strains, and the extract of HQD1 exhibited antimicrobial activity against MRSA with an MIC value of 0.031 mg/mL, which was in accordance with the previous reports of Rivai et al. RM (11 isolates, 39.13%) and GM (9 isolates, 19.56%) were more suitable for antitumor agent production, which agreed with previous reports of GM-cultivated Oidiodendron truncatum leading to the discovery of the potent anticancer agent chetracin B with cytotoxicity against five human cancer lines reaching nM degree. The variation in the antibiotic and cytotoxic properties among media could possibly be related to the composition of RM, and GM activated our isolated fungal biosynthetic gene clusters.

Materials and methods

Fungal material used. Previously isolated endophytic fungi from healthy roots, stems, leaves, hypocotyls, and flowers of R. stylosa (No. 201510-HHL) and R. mucronata (No. 201510-HQD) were used for the present study. The titled plant materials were collected from a specific location (110°32′–110°37′ E, 19°51′–20°01′ N) under the permission of Dong Zhai Gang Mangrove National Nature Reserve Authority of Hainan Island, China. Based on the consideration on the conservation of mangrove plants resources, we had to collect only a few individuals per species. The study’s authors promise the use of plants in the present study complies with international, national and/or institutional guidelines. These endophytic fungi were identified in combination with morphologic characteristics and p internal transcribed spacer (ITS) sequences. Fungal ITS-rDNA sequences of 46 representative isolates were deposited in GenBank under the accession number KX618209 and numbers ranging from KX631698 to KX631742, and their antioxidant capacity has also been demonstrated.
**Fermentation and extraction.** Fungal isolates were cultured on Petri dishes of potato dextrose agar (PDA) at 28 °C for 5 days and then inoculated on four different media, PDA, Czapek’s agar (CZA), rice medium (RM), and grain medium (GM), for stationary fermentation. After 40 days, the harvested cultures were extracted with 100 mL of ethyl acetate, ultrasonicated at 50 °C for 1 h, and then filtered through filter paper. A rotary evaporator was used at 50 °C with low pressure to evaporate the remaining ethyl acetate. The extracts were dissolved in dimethyl sulfoxide (DMSO, not exceeding 1%, v/v) and stored at 4 °C until use.

**Screening for antimicrobial activity.** The endophytic fungal extracts were tested against the gram-negative bacterium *Pseudomonas adageae* (PA), gram-positive bacteria *Enterococcus faecalis* (EF), methicillin-resistant *Staphylococcus aureus* (MRSA) and pathogenic fungus *Monilia albicans* (MA) by the microdilution method with some modifications. An aliquot of extract (2 μL) was added to 198 μL of the indicator strain suspension at a density of 5 × 10^6 CFU/mL into each well of a 96-well microplate. This mixture was then incubated for 24 h/48 h at 37 °C/28 °C, and the minimum inhibitory concentration (MIC) was recorded. Ciprofloxacin was used as a positive control for bacteria, and amphotericin B was used as a positive control for fungi.

**Screening for cytotoxicity.** Cytotoxicity was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method according to our previous report. Human cervical carcinoma cells (HeLa), human lung cancer cells (A549), and human hepatoma cells (HepG2) were purchased from Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and the cell lines were grown in RPMI-1640 culture medium with 200 μL/mL foetal bovine serum (FBS) under a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. A 100 μL cell suspension at a density of 1.5 × 10^5 cell mL⁻¹ was pipetted into 96-well microtiter plates. Fungal extracts with different concentrations from 100 to 600 μg/mL were added to each well and incubated for 24 h under the above conditions in a CO₂ incubator. Then, 20 μL of MTT (5 mg/mL) was added to each well, and the plates were further incubated for 3 h. DMSO (200 μL) was added to dissolve the formazan crystals. The absorbance was then measured at 570 nm by a microplate reader. The cell inhibition rate (%IR) was calculated by the following equation: % IR = ([A_{test} - A_{control}]/A_{control}) × 100, where A_{test} is the absorbance of the blank and A_{control} is the absorbance of the test compounds. The IC₅₀ value was calculated from the dose-response relationship. Doxorubicin was used as the positive control.

**The profiling of secondary metabolites by HPLC.** To assign the bioactivities of the endophytic fungal cultural extract, we performed profiling of the compounds with a Waters 2998 series HPLC system. Separation was achieved using a 250 mm column at 26 °C with a multistep linear gradient elution program in which chromatographic methanol changed from 0 to 20% in 5 min, from 20 to 25% in 5–15 min, from 25 to 50% in 15–30 min, from 50 to 65% in 30–40 min, from 65 to 85% in 40–50 min, and finally from 85 to 100% in 50–60 min. UV spectra were recorded at 254 nm. The extraction of rice medium was used as a control.

**Statistical analyses.** SPSS 23.0 software was used for statistical analysis. Ordinary one-way ANOVA and principal component analysis (PCA) used for the different measured variables in the study. Significance was evaluated in at a level of P < 0.05, for the endophyte extract of different medium to antimicrobial (PCA) and cytotoxic activities (ANOVA) were performed using Tim Duncan’s test. All errors are expressed as standard deviations (SD).

**Conclusion**

To date, there are few systematic studies on the antimicrobial and antitumour potential of mangrove endophytic fungi. Our study indicated that the antimicrobial and cytotoxic activities of mangrove endophytic fungal extracts grown on four media showed distinguishable differences in activities and revealed that RM could promote the secretion of bioactive secondary metabolites. *Neopestalotiopsis protearum* HQD5 and *Pestalotiopsis sp.* HQD6 showed potent antimicrobial activity; *Neopestalotiopsis protearum* HHL46, *Phomopsis longicolla* HHL50, *Botryosphaeria fusispora* HQD83, *Fusarium verticillioides* HQD48 and *Pestalotiopsis sp.* HQD6 displayed significant antitumour activity. Considering these results, these fungi could be further explored for the characterization of antimicrobial and cytotoxic secondary metabolites, which could explain the significant biological activities of the abovementioned fungal strain.

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