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Original Research Article

Isolation, Characterization and Anti-Multiple Drug Resistant (MDR) Bacterial Activity of Endophytic Fungi Isolated from the Mangrove Plant, *Aegiceras corniculatum*

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Abstract

Purpose: *To isolate, characterize and assess the anti-multiple-drug resistant (MDR) bacterial potential of culturable endophytes from A. corniculatum in Beibu Gulf, China.*
Methods: *The plant parts were collected from healthy-looking A. corniculatum. The endophytes were isolated and identified by colonial morphology and macroscopic characteristics and DNA sequencing of fungal ITS region, and then grouped by phylogenetic analysis. Antibacterial activity was assayed using five human pathogenic bacteria (B. cereus, P. aeruginosa, E. coli, K. pneumoniae and A. baumanii) out of which two of them were MDR bacteria. Ethyl acetate extracts from endophytes were prepared, and the minimum inhibitory concentration (MIC) of 3 endophytes was tested using serial 2-fold dilutions of the extract.*
Results: *61 endophytes obtained from A. corniculatum were grouped into 6 genera (Colletotrichum, Alternaria, Phomopsis, Pestalotiopsis, Guignardia, Cladosporium). Colletotrichum and Pestalotiopsis were the most frequent genera, accounting for colonization frequencies (CF) of 29.5 and 37.7%. Among the rare morphotypes, Alternaria, Phomopsis, Guignardia and Cladosporium were the infrequent genera, accounting for CF ranging from 3.2 to 13.1%. Overall, 3 endophytes, including Glomerella, Guignardia, and Cladosporium, all isolated from the leaves, showed inhibitory activity against five test bacteria in vitro. The endophyte, Colletotrichum, inhibited two MDR K. pneumoniae and A. baumanii, while Guignardia inhibits MDR K. pneumoniae. The MIC of the extract of Colletotrichum against MDR K. pneumoniae was 4 µg/ml, against MDR A. baumanii was 0.5 µg/ml, while MIC of Guignardia to K. pneumoniae was 8 µg/ml.*
Conclusion: *The study demonstrates that endophytes from mangrove plant A. corniculatum were a fascinating fungal reservoir against MDR pathogenic bacteria.*

Keywords: *Endophytic fungi, Multiple drug-resistant Bacteria, Mangrove plant, Aegiceras corniculatum*

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INTRODUCTION

Aegiceras corniculatum is a species of mangrove shrub or tree with a distribution in coastal and estuarine areas. Due to living in specifically marine habitats, *A. corniculatum* can be used in various traditional medicinal systems for the treatment of rheumatism, painful arthritis, nociceptor, bacterial infection and inflammation [1-3]. Endophytes are fungi that live for all or part of their life cycle internally and asymptomatically within plant parts [4]. Some endophytes have

proved to produce the same or similar chemical compounds as those obtained from their host with antibiotic, antiviral and anticancer activity [5-6]. Endophytes derived from mangrove plant have become interesting as significant resources for new chemicals in drug discovery [7-9].

Now, multiple-drug resistant (MDR) bacteria are a big public health problem in our world [10]. Among these, the multidrug-resistant (MDR) *K. pneumoniae* and *A. baumanii* are already emerging as causes of several hospital outbreaks in various geographic areas [11-13]. It is really urgent to improve the current strategies to control this global public health threat.

In the present work, fungal endophytes from stems and roots and leaves of *A. corniculatum* were isolated and identified. These fungal endophytes were identified using a combination of morphological and ITS-sequence based molecular methods. Characterization and anti-MDR *K. pneumoniae* and *A. baumanii* activity of the endophyte assemblages were also evaluated. This study will provide data on the characterization and anti-MDR bacteria activity of endophytes associated with Rhizophoraceae mangrove *A. corniculatum*.

EXPERIMENTAL

Plant material

The plants were located in the mangrove forest areas of Qinzhou, Beibu Gulf, Guangxi Province, China. The mean annual temperature is 21.4 – 22.0^{o}C and the mean annual precipitation is about 1649.1 - 2055.7 mm.

Isolation and culture of the endophytes

For endophytes isolation, healthy plant segments were selected from *A. corniculatum* at random, and washed in running tap water. The plant parts were surface sterilized with 70% ethanol for 3 min followed by 1% sodium hypochlorite for 2 min. Surface sterilized plant parts were dried, cut into 0.5-cm lengths, and transferred to Petri dishes containing potato dextrose agar (PDA) medium (50 µg/ml Streptomycin and 100 µg/ml Ampicillin), after taking imprint of dried sterile plant parts. These plates were incubated at 26^{o}C for 5 – 10 days. Hyphal tips of the developing fungal colonies were immediately transferred into fresh PDA agar plates to get pure culture.

Identification of endophytes

All isolates were grouped by observing their colonial morphology and macroscopic characteristics, such as shape, size, color and surface texture, and microscopic features, such as the size and shape of hyphae, conidia and conidiophores. The remaining fungi were subjected to identification of molecular method. Total genomic DNA of the endophytes was extracted (DNeasy plant minikit, Qiagen) according to manufacturers' protocol. ITS1-5.8S-ITS2 region of rDNA was amplified with universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The PCR mixture (50 µl) consisted of 100 ng genomic DNA, 5 µl of 10 × PCR buffer, 1.5 µM $MgCl_2$, 0.5 µM each primer, 200 µM of each dNTP, 1 unit Taq polymerase and autoclaved double-distilled water. PCR were performed with a pre-heating at 95°C for 2 min, followed by 35 cycles at 94°C for 1 min, at 52°C for 1 min, at 72°C for 1 min, and at 72°C for 10 min. The PCR products were sequenced by the service of Beijing Sanboyuanzhi Company Ltd (Beijing, China). The ITS sequences of representative isolates have been submitted to NCBI GenBank database with accession no: KC145168, KC145169, KC145170, KC145171, KC145172, KC145173, KC145174, KC145175, KC145176, KC145177.

Phylogenetic analysis of endophytic fungi

The ITS sequences of the endophytes were used as query sequence to search for similar sequences from GenBank using BLASTn program. The resulting sequences were aligned with ClustalX software, and gaps treated as missing data. To construct the relevant phylogenetic tree, MEGA 5.0 software was employed. The alignment data were subsequently analyzed by the neighbor-joining (NJ) method (Kimura two-parameter distance calculation). The bootstrap was 1,000 replications to assess the reliable level to the nods of the tree.

Determination of antibacterial activity and minimum inhibitory concentration

All endophytes were tested for antimicrobial activity against five bacterial strains *Bacillus cereus* (ATCC 10876), *Pseudomonas aeruginosa* (ATCC 27853), *E. coli* (ATCC 25947), *Klebsiella pneumoniae* and *Acinetobacter baumanii* by K-B method according to American NCCLS statute (the susceptibility of *A. baumannii* and *K. pneumoniae* to selected antibiotics were demonstrated in Table 1). The broth of each liquid cultured

Table 1: Susceptibility of tested strain *A. baumannii and K. pneumoniae* to selected antibiotics

Strain	Antibiotic									
	PIP	***GEN***	***CIP***	***IMP***	***LVX***	***SAM***	***CTX***	***FEP***	***SXT***	***AMK***
A. baumannii	+	+	+	-	+	-	+	+	-	+
K. pneumoniae	-	+	+	-	+	-	+	+	+	+

Note: *PIP, Piperacillin; GEN, gentamicin; CIP, ciprofloxacin; IMP, imipenem; LVX, levofloxacin; SAM, ampicillin-sulbactam; CTX, Cefotaxime; FEP, Cefepime; SXT, trimetropim-sulfamethoxazole; AMK, Amikacin; + = antibiotic-resistant; - = antibiotic-sensitive*

endophytes were filtered through filter paper, and the sterilized paper discs impregnated with supernatant were placed on the surface of the LB medium already seeded with test bacterium in Petri Plate. The plates were incubated at 37±2°C for 24 h and measured inhibition zones.

To assess minimum inhibitory concentration (MIC), the endophytes were inoculated into 500-ml Erlenmeyer flasks containing 250 ml of the PDB and cultured at 150 rpm at 26°C for 10 days in a rotary shaker. The contents were mixed thoroughly with Glass rod, and then filtered. The filtrate was extracted thrice with ethyl acetate at 60°C and filtered. The combined filtrates were evaporated to dryness under reduced pressure on rotary evaporator. Next, the dry ethyl acetate extract were dissolved in 10 ml of methanolic. Serial 2-fold dilutions of the extract were used to test the MIC. The extract was incorporated into nutrient agar at concentrations of 0.5 µg/ml, to 128 µg /ml. A control without the extract was also set up. 500 µl each of the test organisms, previously diluted to give 4×10^4 cfu/ml was used to inoculate the plates. These were incubated at 37 ± 2°C for 24 h. The MICs of the extract were recorded by reading the lowest concentration that inhibited visible growth.

Statistical analysis

Each parameter was tested in triplicate. All data were analyzed using statistical analysis software SPSS13.0 (Statistical Product and Service Solutions, Inc, Chicago, IL, USA). $p > 0.05$ was set as the significant threshold for all statistical analysis.

RESULTS

Characteristics of culturable endophytes

Epiphytic fungi of plant segments were efficient eliminated by surface disinfection treatments and the segments imprint yielded no fungi and bacteria. Sixty one endophytes were isolated from 152 tissue segments of *A. corniculatum* grown in the Qinzhou of Guangxi beibu Gulf, China. Ten different fungi were isolated based on the colonial morphology and macroscopic characteristics (Fig 1).

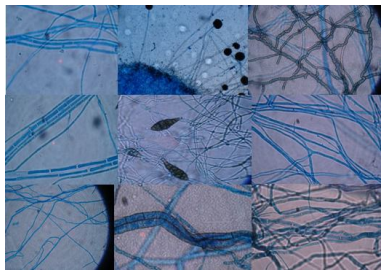

Fig 1: Light micrographs of endophytes isolated from *A.corniculatum*

To confirm the reliability of morphological identification, all 10 morphotypes were subjected to molecular identification based on ITS sequence analysis. Table 2 shows the percent similarity of these isolates with the fungi identified and their respective accession number obtained from GenBank. Seventy endophytes were isolated from the leaves and, 17 from the roots and 27 from the stems.

Table 2: Culturable endophytic fungal ITS sequences closely matching GenBank sequences

Isolate no.	Accession no. from present	Genbank closest match	Accession number	Sequence coverage (%)	Max. identity (%)
CYP1	KC145168	Fungal sp.	HM211235	98	99
CYP2	KC145169	*Alternaria* sp.	JN038451	98	99
CYP3	KC145170	*Phomopsis phyllanthicola*	JN107737	97	99
CYP4	KC145171	*Pestalotiopsis* sp.	EU644755	98	98
CYP5	KC145172	*Pestalotiopsis* sp.	JF502635	99	99
CYP6	KC145173	*Alternaria* sp.	JN038451	96	99
CYP7	KC145174	*Guignardia mangiferae*	EU677803	98	99
CYP8	KC145175	*Phomopsis* sp.	FJ487921	97	99

In addition, the stems of *A. corniculatum* were colonized by a greater number of endophytes relative to leaves and roots. It seems likely that stem tissues may be better for colonization. Tissue specificity of endophytes was also observed. For example, *Guignardia* sp. and *Cladosporium* sp. colonized leaves exclusively, and *Alternaria* sp., *Phomopsis* sp. can only colonized stems and roots (Fig. 2).

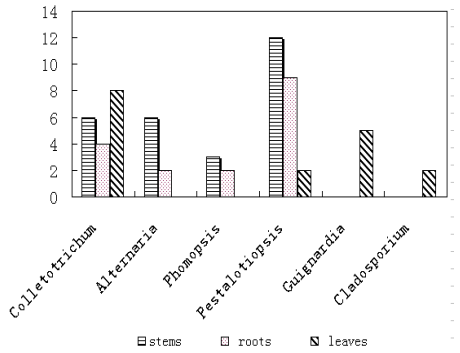

Fig 2: The frequency of 6 different ITS-based genotypes determined from total endophytes. Genus and/or species names of identified fungi are indicated above the corresponding column.

Analysis of distribution frequencies of the 61 endophytes revealed that the fungal communities in the host contained a few frequent genera and infrequent groups. *Colletotrichum* and *Pestalotiopsis* were the frequent genera, accounting for colonization frequency 29.5 and 37.7%, respectively. Among the rare morphotypes, *Alternaria*, *Phomopsis*, *Guignardia*, *Cladosporium* were the infrequent genera, accounting for colonization frequencies ranging from 3.2 to 13.1%. Phylogenetic relationship of these isolates with their related fungi is shown in Fig 3. All the isolated endophytes belong to phylum Ascomycota.

Molecular phylogenetics

The ITS neighbor-joining tree of the endophytes is shown in Figure 3. The 10 morphospecies (CYP1, CYP2, CYP3, CYP4, CYP5, CYP6, CYP7, CYP8, CYP9, CYP10) sharing sequence max identity of ≥ 98% with available data in NCBI (Table 2) were grouped into 6 genera of *Colletotrichum*, *Alternaria*, *Phomopsis*, *Pestalotiopsis*, *Guignardia*, *Cladosporium*. Among these endophytes, the strains CYP2, CYP4, and CYP9 were located with high

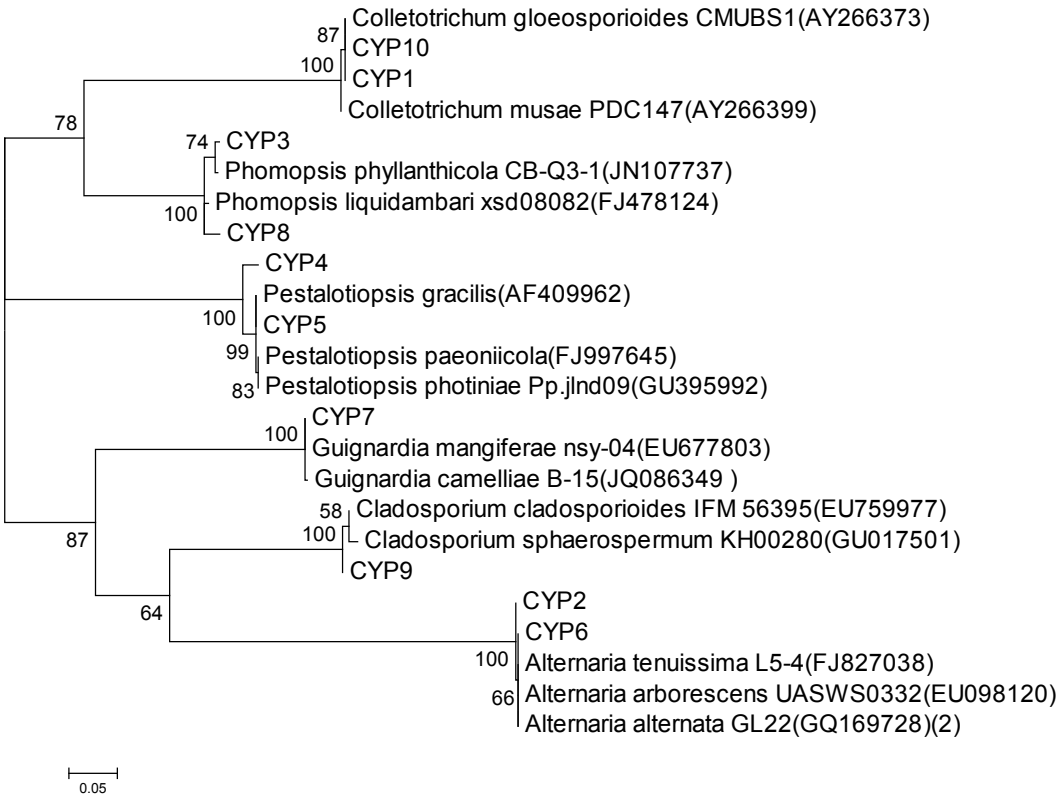

Fig 3: Neighbor-joining tree of the ITS sequences of the endophytes associated with *A.corniculatum.* The tree was constructed based on rDNA sequence (ITS1, 5.8S and ITS 4) by using neighbor-joining method. The bootstrap consensus tree inferred from 1,000 replicates.

Table 3: Antibacterial activity of liquid media cultured endophytes

Tested Strain	CYP7		CYP9		CYP10	
	IZ	*IA*	*IZ*	*IA*	*IZ*	*IA*
B. cereus	15.32±0.45	++	16.07±0.30	++	13.76±0.30	++
P. aeruginosa	6.00±0.00	-	6.00±0.00	-	6.00±0.00	-
E. Coli	6.00±0.00	-	8.22±0.15	+	9.07±0.32	+
K. peneumoniae	14.57±0.37	++	6.00±0.00	-	17.56±0.51	++
A. baumanii	6.00±0.00	-	6.00±0.00	-	24.16±0.42	+++

Note: IZ: diameter of inhibition zone; IA: inhibition ability, (+) =6-10 mm; (++) =10–20 mm; (+++) > 20 mm; (−) =No activity means in each column having the same superscript letters are not significantly different at $p \leq 0.05$.

Colletotrichum gloeosporioides CMUBS1 (AY266373) formed a clade with 87% bootstrap support. Strains CYP2, CYP3, CYP4, CYP5, CYP6, CYP7, CYP8, CYP9 and CYP10 shared sequence max identities from 97 to 98% with *Alternaria alternata* (100% bootstrap), *Phomopsis phyllanthicola* (85% bootstrap), *Pestalotiopsis malicola* (100% bootstrap), *Pestalotiopsis photiniae* (85% bootstrap) , *Alternaria alternata* (100% bootstrap), *Guignardia mangiferae* (70% bootstrap), *Phomopsis liquidambari* (59% bootstrap), *Cladosporium cladosporioides* (100% bootstrap) and *Colletotrichum gloeosporioides* (87% bootstrap) respectively.

Antimicrobial activity

In continuation of searching isolates with anti-MDR bacterial activity, all six endophytes were selected to test their antibacterial activity against 5 potent human bacterial pathogens (*B. cereus, P. aeruginosa, E.coli, K. pneumoniae, A. baumanii*) by K-B method. In the five tested strain, *K. pneumoniae* and *A. baumanii*, which were isolated from patient with serious respiratory tract infection, were found to be MDR pathogenic bacteria against several different kinds of antibiotics (Table 1). Out of the six endophytes, three (50%) (*Colletotrichum, Guignardia*, and *Cladosporium*) inhibited the growth of at least one or more bacterial pathogens. *Colletotrichum* inhibited the growth of 4 out of 5 tested human pathogens (80%) and two were MDR bacteria (*K. pneumoniae* and *A. baumanii*), which was significantly different at $P_{0.05}$ level (Table 3). Endophyte CYP7 (identified as *Guignardia* sp.) could also inhibit the growth of 2 out of 5 tested bacterial strains and one was MDR bacterium (*K. pneumoniae*), which was significantly different at $P_{0.05}$ level. Endophyte CYP9 (identified as *Cladosporium* sp.), showed high activity against *B. cereus* and *E. coli*, which was significantly different at $P_{0.05}$ level.

The MICs of the extract against the test organisms are shown in Table 4. The MIC of CYP7 was 4.0 µg/ml against standard strains of *B. cereus*; 8 µg/ml against MDR clinical isolate *K. pneumoniae*. The MIC of CYP9 was 4.0 µg/ml against standard strains of *B. cereus*; 32 µg/ml against standard strains of *E. coli*. The MIC of CYP10 was 4.0 µg/ml against *B. cereus*, 16 µg/ml against *E. coli*, 4 µg/ml against *K. pneumoniae*, and 0.5 µg/ml against *A. baumanii*. Control did not produce any inhibitory activity against the organisms. The MIC of the crude extract for some tested strains was not determined, since there was no inhibitory activity in the preliminary test.

Table 4: Minimum inhibitory concentrations (MICs) of ethyl acetate total extract from 3 endophytes

Test strain	* MIC (µg/ml)		
	CYP7	*CYP9*	*CYP10*
B. cereus	4	4	4
P. aeruginosa	ND	ND	ND
E. Coli	ND	32	16
K. peneumoniae	8	ND	4
A. baumanii	ND	ND	0.5

** = mean of 3 determinations; ND = not determined since there was no inhibitory activity*

DISCUSSION

Since the antibiotic overuse, multiple-drug resistant (MDR) bacteria have been reported as a big public health problem today. Most studies define multidrug resistance as resistance to more than two classes of antibiotics [14]. Many human pathogenic bacteria are examples of this emerging crisis, and they are extremely difficult to treat with present existing extended-spectrum antibiotics, leading to increased morbidity and mortality in human [15]. New antibacterial compounds from mangrove endophytes may be an extractive way to solve this problem.

In the present work, endophytes *Colletotrichum* sp., *Alternaria* sp., *Phomopsis* sp., *Pestalotiopsis* sp., *Guignardia* sp., and *Cladosporium* sp. from *A. corniculatum* were isolated. Three endophytes; *Glomerella* sp., *Guignardia* sp., and

Cladosporium sp., all isolated from leaves, showed inhibiting activity against five tested bacteria in vitro. Two of them inhibit one or two MDR *K. pneumoniae* and *A. baumanii* with comparably lower MICs, that showing promising application in treating MDR bacteria.

Many species in *Colletotrichum* are plant pathogens, although some species are endophytes living in non-disease hosts [16]. Huang found that pathogenic strains, which result in the anthracnose of mangrove along the coast of beibu Gulf in Guangxi from 1996 to 1997, belong to *Colletotrichum gloeosporioides* [17]. In our work, *Colletotrichum* was found to be one of the frequent genera, but all the strains was isolated from healthy stems and leaves and were found as asymptomatic endophytes. Our isolated strains may belong to opportunistic pathogenic fungi or may represent a genetically distinct population of *C. gloeosporioides*.

Another attractive finding about this isolated *Colletotrichum* was its antibacterial activity against four bacterial pathogenic strains and two are MDR bacteria (*K. pneumoniae* and *A. baumanii*). Zou found a new antimicrobial metabolite, named colletotric acid, from a liquid culture of *C. gloeosporioides*, an endophyte found inside the stem of *Artemisia mongolica* [18]. Further purification and chemical analyses are warranted to elucidate the structure of the antibacterial compound. *Alternaria* species are also known as major plant pathogens.

Not all *Alternaria* species are pathogens, some have shown promise as biocontrol agents against invasive species. In our present study, *Alternaria* was also found to be frequent genera (13.1%) and show no infectious symptom to its host. *Phomopsis* is an important mangrove endophyte, and many significant new bioactive metabolites were isolated from this fungus [7,9,19,20]. *Pestalotiopsis* species are enormously distributed all over the world, occurring common in tropical and temperate ecosystems [21] and many isolated as endophytes [22]. Xing investigated four *Rhizophoraceae* mangrove plant species on the south coast of China and found that *Pestalotiopsis* was one of the most frequent endophyte [23]. More than 130 different compounds have been isolated from various species of *Pestalotiopsis* [24].

Guignardia is a genus of fungi in the family *Botryosphaeriaceae*. Many species of *Guignardia* have been isolated from corresponding host plants and some are important source of novel secondary metabolites [25-27]. In the present study, species of *Guignardia* has shown antibacterial activity against *B. cereus* and *K. pneumoniae*. *Cladosporium* species are *Deuteromycete* fungi and have a worldwide distribution [28]. Several researchers have found that *Cladosporium* have some potential bioactivity, such as antibacterial [29] or antifungal activity [30]. In the present study, species of *Cladosporium* has shown antibacterial activity against *B. cereus* and *E. coli*.

CONCLUSION

The overall findings of this study show that endophytes isolated from the host, *A. corniculatum*, exhibited promising anti-MDR bacteria activity against human pathogens.

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